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PRINCIPAL INVESTIGATOR: Lewis A. Chodosh, M.D., Ph.D.

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Philadelphia, Pennsylvania 19104-3246

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Lewis A. Chodosh, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

E-MAIL:
chodosh@mail.med.upenn.edu

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13. ABSTRACT *(Maximum 200 Words)*

Germline mutations in the breast and ovarian cancer susceptibility gene, *BRCA1*, account for a large proportion of families with inherited breast and ovarian cancer. Interestingly, while germline *BRCA1* mutations predispose carriers to adenocarcinoma of the breast, no somatic *BRCA1* mutations have been found in sporadic primary breast cancers. This observation suggests that this molecule may normally protect the breast against carcinogenesis only during specific stages of mammary gland development. Previously, we have analyzed the temporal and spatial pattern of *Brca1* expression during normal mouse embryogenesis, in adult tissues, and during postnatal mammary gland development. These studies support a role for *Brca1* in the regulation of cell proliferation and differentiation in the breast during puberty and pregnancy. We hypothesize that *Brca1* plays a critical role in mammary gland development, and that its function is temporally restricted to particular developmental phases. In this application, we propose to test this hypothesis by using a modified tetracycline-inducible expression system to either induce or abolish *Brca1* expression in transgenic mice during particular developmental stages in a temporally-restricted and breast-specific manner. Through this approach, we hope to understand more clearly how the loss or mutation of this molecule contributes to carcinogenesis in a developmental-specific manner.

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Lewis L. Moody MD PhD)
10/14/99

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INTRODUCTION

Genetic analysis of families in which multiple individuals have developed breast cancer suggests that 5-10% of breast cancer cases result from the inheritance of germline mutations in autosomal dominant susceptibility genes[1, 2]. Germline mutations in one of these breast cancer susceptibility genes, *BRCA1*, appear to account for most families with inherited breast and ovarian cancer, and somewhat less than half of families displaying inherited breast cancer alone[3, 4]. The *BRCA1* gene encodes a 220 kDa phosphoprotein that contains a RING finger motif, a transcriptional activation domain, and a BRCT domain typically found in proteins involved in cell cycle regulation and DNA damage response[4-10]. Tumors arising in patients with germline *BRCA1* mutations almost invariably display loss of the wild-type *BRCA1* allele, suggesting that *BRCA1* is a tumor suppressor gene[11]. Taken together with findings that reduction in *BRCA1* expression *in vitro* results in accelerated growth of breast and ovarian cancer cell lines, and that overexpression of *BRCA1* results in inhibited growth of such cell lines, these observations are consistent with a model in which *BRCA1* negatively regulates proliferation in adult tissues[12-14]. Interestingly, however, we have found that the murine homologue of *BRCA1* is expressed at highest levels in the mouse in cellular compartments containing rapidly proliferating cells undergoing differentiation, such as are found in the breast during puberty and pregnancy[15, 16]. The positive correlation between *Brca1* expression and cellular proliferation may be explained in part by the observation made in several laboratories, including our own, that the expression of this gene is regulated in a cell cycle-dependent manner with peak steady-state levels of mRNA and protein occurring just prior to and during S-phase[5, 17-19]. The discovery that BRCA1 is phosphorylated in a cell cycle-dependent fashion, as well as the finding that *BRCA1* may be a substrate for certain cyclin-dependent kinases, suggests a possible function for *BRCA1* in cell cycle progression and the regulation of proliferation[5, 20]. This hypothesis is supported by reports that *BRCA1* overexpression inhibits cell cycle progression at least in part by upregulating expression of *p21*$^{WAF1/CIP1}$, a cyclin-dependent kinase inhibitor that contributes to the growth arrest response to DNA damage [21, 22]. Interestingly, recent studies have demonstrated that the BRCA1 protein forms a complex with Rad51[23]. Since Rad51 is required for the proper response to ionizing radiation in yeast, these studies suggest a role for BRCA1 in the response to DNA damage. The observation that BRCA1 is rapidly phosphorylated in response to DNA damage, including that caused by ionizing radiation, strongly supports this model[24, 25]. It is important to note, however, that studies of tumor suppressor genes such as *Rb* and *p53* and have highlighted the fact that proteins in this class typically function in multiple pathways and processes in the cell.

Interestingly, while germline *BRCA1* mutations predispose carriers to adenocarcinoma of the breast, no somatic *BRCA1* mutations have been found in sporadic primary breast cancers. This observation suggests that this molecule may normally protect the breast against carcinogenesis only during specific stages of mammary gland development. Previously, we have analyzed the temporal and spatial pattern of *Brca1* expression during normal mouse embryogenesis, in adult tissues, and during postnatal mammary gland development. These studies support a role for *Brca1* in the regulation of cell proliferation and differentiation in the breast during puberty and pregnancy. We hypothesize that *Brca1* plays a critical role in mammary gland development, and that its function is temporally restricted to particular developmental phases. The focus of this project is to test this hypothesis by using a modified tetracycline-inducible expression system to either induce or abolish *Brca1* expression in transgenic mice during particular developmental stages in a temporally-restricted and breast-specific manner. The goal of this work is therefore to understand more clearly how the loss or mutation of this molecule contributes to carcinogenesis in a developmental-specific manner. This goal will be accomplished by pursuing the following specific aims:

Specicic Aim 1. Develop breast-specific, tetracycline-dependent expression systems to inducibly overexpress or abolish *Brca1* expression in the mammary epithelium *in vivo*. We will develop a modified tetracycline-inducible expression system in order to conditionally express *Brca1* or antisense *Brca1* mRNA in the breast in a temporally-restricted manner. Constructs will be generated in which the expression of the reverse tetracycline transcriptional activator, rtTA, will be breast-specific and dependent upon the presence of tetracycline. Since both the expression and function of rtTA are tetracycline-dependent, target expression constructs can be developed in which wild-type or mutant *BRCA1* cDNA clones are expressed in a tetracycline-dependent manner by appending them to *Tn10* operator-containing promoters. Additional target expression constructs will be developed in which *Brca1* antisense RNA is expressed in a tetracycline-dependent manner. Transgenic mouse lines will be generated which overexpress the reverse tetracycline transcriptional activator, rtTA, in a breast-specific manner from the mouse mammary tumor virus LTR. Transgenic mouse lines will also be generated which overexpress the reverse tetracycline transcriptional activator, rtTA, in a tetracycline-dependent manner. Finally, transgenic mouse lines will be generated which contain target DNA constructs that direct the tetracycline-dependent expression of wild-type or mutant *Brca1*, or *Brca1* antisense RNA. These transgenic strains should permit the inducible expression of target transgenes during specific stages of mammary gland development.

Specific Aim 2. Inducibly overexpress *Brca1* in the mammary epithelium of transgenic mice during specific developmental stages. The effect of overexpressing *Brca1* during specific stages of mammary gland development will be determined. Bitransgenic mice which express both the rtTA tetracycline-dependent transcriptional activator and a *Brca1* wild-type or mutant transgene driven by a tetracycline-dependent promoter will be derived by mating each of the transgenic strains developed in specific aim 1. *Brca1* overexpression in bitransgenic mice will be induced during specific stages of mammary gland development, including puberty, pregnancy, lactation and regression, by tetracycline treatment during the appropriate developmental window. Glands will be analyzed by morphological and molecular methods for abnormalities in mammary epithelial proliferation, differentiation and development, and for signs of hyperplasia, dysplasia and neoplasia.

Specific Aim 3. Inducibly abolish *Brca1* expression in the mammary epithelium of transgenic mice during specific developmental stages. The effect of abolishing *Brca1* expression during specific stages of mammary gland development will be determined by creating bitransgenic mice which express both the tetracycline-dependent transcriptional activator, rtTA, and a *Brca1* antisense transgene driven by a tetracycline-dependent promoter by mating the transgenic strains developed in specific aim 1. Reduction of *Brca1* expression in bitransgenic mice will be induced during specific stages of mammary gland development as above. Glands will be analyzed by morphological and molecular methods for abnormalities in mammary epithelial proliferation, differentiation and development, and for signs of hyperplasia, dysplasia and neoplasia.

BODY

Technical Objective 1: Develop breast-specific, tetracycline-dependent expression systems to inducibly overexpress or abolish BRCA1 expression in mammary epithelial cells.

Task 1: Construct vectors for expressing rtTA and/or tTA in the mammary epithelium.

In order to create an inducible expression system in mammary epithelial cells *in vivo*, we have constructed several mammary-specific and tetracycline-dependent expression vectors. Specifically, we have made use of the reverse tetracycline-controlled transcriptional activator, rtTA, that fuses the herpes simplex virus VP16 transcription activation domain with a mutant form of the DNA binding domain from the tet repressor of *E. coli*[26]. This transactivator requires tetracycline derivatives for specific DNA binding. Target genes are placed under the control of the tetO regulatory cassette from the tetracycline-resistance operon of Tn10. This system has been documented to rapidly induce gene expression in the presence of tetracycline by up to three orders of magnitude with a low level of basal expression. Moreover, the availability of numerous tetracycline analogs with varied binding affinities, as well as the use of varying concentrations of tetracycline, permits the absolute level of transgene expression to be reproducibly and precisely titrated. As such, this system is ideally suited for the tight control and rapid induction of potentially toxic genes to desired levels of expression.

Plasmid pUHD172-1neo was constructed by Gossen et al. and contains a neomycin-selectable marker as well as sequences encoding the reverse tetracycline-controlled transcriptional activator, rtTA, whose expression is driven by a constitutive CMV promoter/enhancer[26] .

Plasmid pUHD15-1 was also constructed by Gossen et al. and contains a neomycin-selectable marker as well as sequences encoding the tetracycline-controlled transcriptional activator, tTA, whose expression is driven by a constitutive CMV promoter/enhancer[26] .

Plasmid pMMTV.rtTA contains sequences encoding the reverse tetracycline-controlled transcriptional activator, rtTA, whose expression is driven by the mouse mammary tumor virus promoter/enhancer and was constructed by replacing the CMV promoter/enhancer of pUHD172-1neo with the entire MMTV promoter/enhancer long terminal repeat (LTR) containing 2.0 kb of upstream sequence. The MMTV LTR is widely used to obtain mammary-specific expression in transgenic mouse model systems.

Plasmid pHMG.rtTA contains sequences encoding the reverse tetracycline-controlled transcriptional activator, rtTA, whose expression is driven by the promoter of the housekeeping gene, hydroxymethylglutaryl CoA reductase (HMG CoA). This plasmid was constructed by replacing the MMTV promoter/enhancer of pMMTV.rtTA with the 2.25 kb NotI/XbaI fragment of pHMG. This fragment contains the mouse HMG promoter, the first noncoding exon, and a portion of the first intron. This construct was generated in order to attempt to direct expression to all cells in the mammary gland, inclusive of epithelial cells and stromal cells.

Task 2: Construct vectors for expressing rtTA and/or tTA in a tetracycline-dependent manner.

Since constitutive expression of rtTA has been reported to be detrimental in some cell types, we have created a modification of the tetracycline-dependent expression system by replacing the constitutive CMV-derived promoter/enhancer driving rtTA expression in pUHD172-1neo, with the tet regulatory sequences (tetO) from Tn10, to create the autoregulatory plasmid, pTetO.rtTA (Fig. 1A). A similar approach has been successfully taken by others with the original tTA tetracycline-repressible transcriptional activator[27]. As a result, in this system the expression of the transcriptional activator, rtTA, is itself induced by the addition of tetracycline, and subsequently induces the target gene in a tetracycline-dependent manner. As a

result, induction occurs at two different regulatory levels - the expression of the rtTA activator, and the binding and activation of the target promoter by rtTA.

Task 3: *Construct target vectors expressing wild type and mutant forms of BRCA1.*

In order to permit the expression of target genes of interest in a tetracycline-dependent manner we have constructed a tetracycline-incucible expression vector, pTet-Target.Puro, that contains both a tetracycline-inducible promoter driving the expression of a target gene, and a puromycin-selectable marker (Fig. 1D). The puromycin-resistance gene, whose expression is driven by the PGK promoter, was obtained from the retroviral vector pLZRS as a ClaI-BspHI fragment, blunted with the Klenow fragment of DNA polymerase, and cloned in the NotI restriction site of pTet-Splice (Life Sciences).

In order to permit the inducible expression of wild-type BRCA1 in mammary epithelial cells in a tetracycline-dependent manner, a full-length cDNA encoding wild-type BRCA1 was subcloned into the HindIII site of pTet-Target.Puro to generate pTet.BRCA1.wt.

In order to permit the inducible expression of mutant forms of BRCA1 in mammary epithelial cells in a tetracycline-dependent manner, a cDNA clone encoding BRCA1 truncated at the carboxy-terminal ApaI restriction site was subcloned into the HindIII site of pTet-Target.Puro to generate pTet.BRCA1.ApaI. In addition, a cDNA clone encoding the naturally occurring mutant of BRCA1 C64G was constructed. This mutation contains a point mutation in the RING finger domain of BRCA1 that has been found to cosegregate with breast and ovarian cancer in BRCA1 families. This point mutation was created by PCR site-directed mutagenesis using overlapping PCR primers containing complementary mutational changes at C64. The resulting HindIII-BglI fragment containing the mutant region was subloned into wild-type BRCA1. The full-length HindIII fragment containing the mutant BRCA1 was then subcloned into the HindIII site of pTet-Target.Puro to generate pTet.BRCA1.C64G.

Task 4: *Construct target vectors expressing BRCA1 antisense RNA.*

In order to create a system in which *Brca1* expression can be inducibly down-regulated, we have used pTet-Target.Puro to construct a tetracyline-inducible target vector, pTetO-Brca1AS, that inducibly expresses *Brca1* antisense RNA complementary to a 322 nucleotide region at the 5' end of murine *Brca1*. This region spans the putative *Brca1* translation initiation codon. A 322 bp region containing the 5' region of mouse BRCA1 was amplified by RT-PCR from first-strand breast cDNA. This region spans the translation initiation codon of BRCA1. This 322 bp PCR fragment was sequenced on both strands to verify that it contained wild-type sequence and was then subcloned into the HindIII-EcoRV site of pTet-Target.Puro in the antisense orientation to generate pTet.BRCA1.AS. As a negative control, the same 322 bp fragment was subcloned into pTet-Target.Puro in the sense orientation to generate pTet.BRCA1.S. This vector should express the same 322 nucleotide fragment in the sense orientiation.

In order to test the ability of the expression vectors described above to permit tetracycline-dependent inducible expression in mammary epithelial cells *in vivo*, we have used these constructs to generate a test inducible expression system in mammary epithelial cells *in vitro*. We have stably transfected pTetO.rtTA, which contains a neomycin-selectable marker, into HC11 mammary epithelial cells to generate the

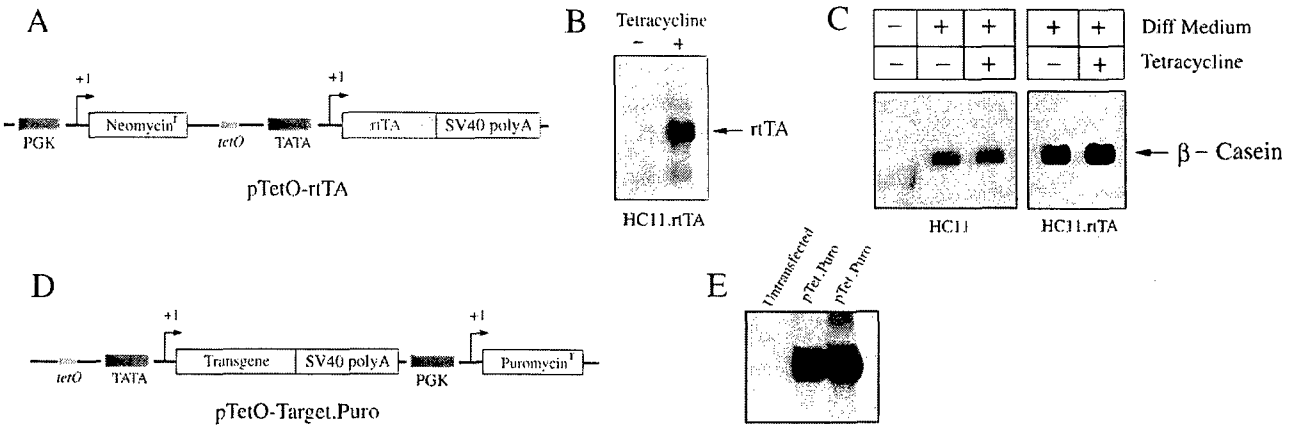

Fig. 1: Generation of a tetracycline-inducible expression system in mammary epithelial cells

mammary epithelial cell clone, HC11.rtTA (Fig. 1A, B). This clone inducibly expresses the rtTA tetracycline-regulated transcriptional activator in the presence of tetracycline (Fig. 1B). In addition, we have demonstrated that the HC11.rtTA clone faithfully differentiates in response to the lactogenic hormones, prolactin, insulin and hydocortisone in the presence or absence of tetracycline, indicating that overexpression of rtTA does not interfere with the process of differentiation (Fig. 1C). Finally, we have demonstrated that induction of HC11.rtTA with doxycycline does not alter proliferation rates in this cell line (not shown). In aggregate, these results demonstrate that the inducible expression of rtTA in mammary epithelial cells represents a suitable system for determining the effect of *Brca1* on mammary epithelial proliferation and differentiation.

In order to test the function of the tetracycline-incucible expression vector, pTet-Target.Puro, that contains both a tetracycline-inducible promoter driving the expression of a target gene, and a puromycin-selectable marker, pTetO-Target.Puro was stably transfected into HC11.rtTA (Fig. 1D). Puromycin-resistant clones obtained following transfection of HC11.rtTA cells with this construct express high levels of mRNA for the puromycin-resistance gene (Fig. 1E). No spontaneously puromycin-resistant clones have been detected following puromycin selection of untransfected HC11.rtTA cells.

In order to test the ability of the tetracycline-dependent transcriptional activator, rtTA, to induce the expression of the tetracycline-dependent target gene in the pTet-Target.Puro expression vector, the parental

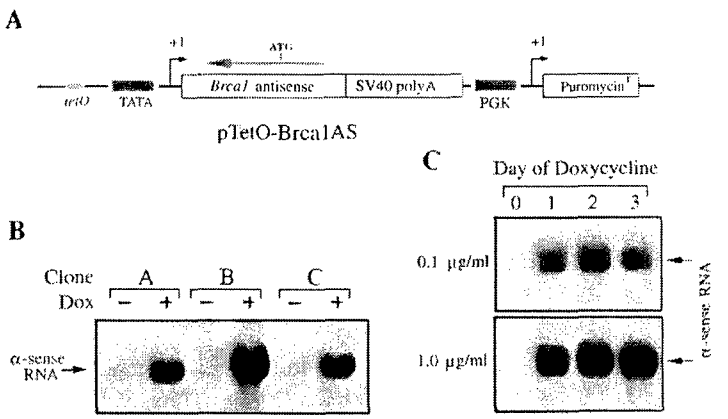

Fig. 2: Graded inducible expression of *Brca2* antisense RNA

HC11.rtTA cell line has been stably transfected with pTet.BRCA1.AS. Puromycin-resistant clones were demonstrated to inducibly express *Brca1* antisense RNA in the presence of doxycycline (Fig. 2B). Induction occurs rapidly, is stable, and occurs in a graded fashion dependent on doxycycline concentration (Fig. 2C). Clones expressing antisense RNA are currently being assayed for their ability to specifically downregulate *Brca1* expression.

Technical Objective II: Create transgenic mice overexpressing BRCA1 or BRCA1 antisense in the mammary epithelium.

Task 1: *Create and identify transgenic lines of mice expressing rtTA and/or tTA in the mammary epithelium.*

In order to test the hypothesis that *Brca1* plays a role in the normal control of mammary epithelial proliferation and differentiation *in vivo*, *Brca1* will be overexpressed in the mammary glands of transgenic mice. Given concerns regarding possible toxic effects of *Brca1* overexpression, we have chosen to conditionally overexpress *Brca1 in vivo* using a tetracycline regulatory system. We have constructed a mammalian expression vector, pMMTV.rtTA, in which expression of the tetracycline-inducible transactivator, rtTA, is driven by the promoter/enhancer of the MMTV LTR. In order to generate transgenic mice harboring this construct, purified DNA containing the pMMTV-rtTA transgene was injected into fertilized oocytes harvested from superovulated FVB female mice. Two founder mice, designated MTA and MTB, were identified that harbored the transgene in tail-derived DNA and that passed this transgene to offspring in a Mendelian fashion.

Surprisingly, Northern hybrization analysis of mammary tissue from a large number of MTA transgenic female mice revealed high levels of expression of rtTA in only 50% of male and female animals harboring the MMTV-rtTA transgene. The remaining 50% of transgenic animals had no detectable rtTA expression. Intriguingly, expression of the rtTA transgene in the line of mice has been found to correlate perfectly with the methylation status of the transgene. Animals harboring an MMTV-rtTA transgene with methylation at HpaII sites near the transcription initiation site do not express rtTA in any tissue, wherease animals bearing unmethylated transgenes express rtTA at high levels. Transgenic parents of a given methylation status are able to give rise to progeny that have both methylated or unmethylated transgenes in the same litter. Thus, this phenomenon is not due to a a classical imprinting mechanism. The nature of this effect is currently being investigated further. Regardless, the fact that MMTV-rtTA transgene-positive animals frequently do not express rtTA, makes the MTA line unsuitable for further studies in the inducible expression of BRCA1.

Characterization of rtTA expression in the MTB line was undertaken. Northern hybrization analysis of mammary tissue from four MTB transgenic female mice and two FVB wild-type controls, revealed high levels of expression of rtTA in all four transgenic female animals (Fig. 3). Expression levels were essentially identical in all four animals. As expected, rtTA expression was not affected by treatment of MTB animals with doxycycline. Unlike the MTA founder line, no transgene-positive animals in the MTB line have been detected that do not express the MMTV-rtTA transgene in mammary tissue. This line was therefore selected for further characterization.

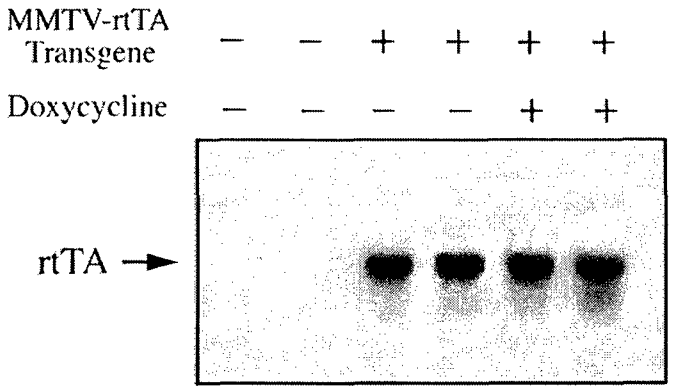

Fig. 3: rtTA expression in MTB transgenic mice

Northern hybridization analysis of 13 tissues derived from male and female progeny of the MTB line of transgenic mice revealed high levels of breast-specific rtTA expression in female virgin animals (Fig. 4). Significant levels of rtTA expression were not detected in other tissues of female mice tested, including brain, skeletal muscle, heart, lung, spleen, kidney, liver, uterus, ovary and testis. rtTA was expressed at low levels in the seminal vesicles of male mice. These results indicated that rtTA is expressed at high levels and in a breast-specific manner in MTB transgenic mice.

We have also constructed mammalian expression vectors, pTetO-LacZ, in which expression of the *LacZ* gene is driven by the tetO-containing promoter cassette, in order to serve as an indicator strain to permit the quantitative characterization of the rtTA/tetO-target bitransgenic expression system created in this proposal. This construct should permit the inducible expression of *LacZ* in response to tetracycline in cells expressing the rtTA transcriptional activator. Using an approach similar to that described above this

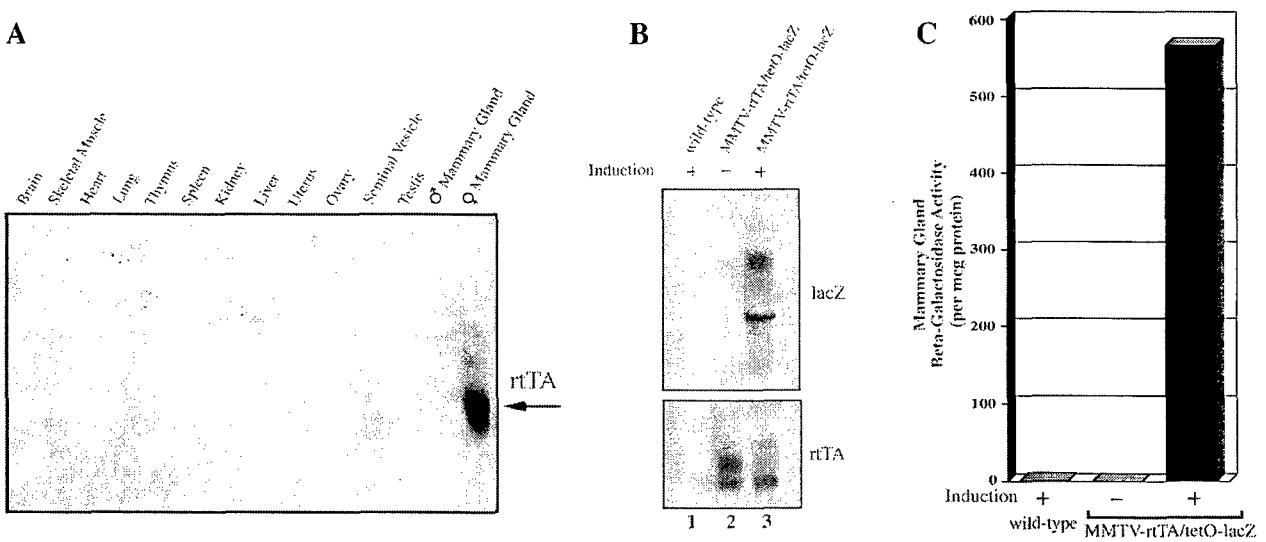

Fig. 4: Bitransgenic system for inducibly overexpressing genes in the breast of transgenic mice

construct, which also contains SV40 splicing and polyadenylation signals, has been used to generate transgenic mice in the FVB background. Founder animals have been identified that pass the pTetO-LacZ transgene to their offspring in a Mendelian fashion.

In order to determine whether this transgenic system will permit inducible transgene expression in the breast in response to tetracycline analogs, MMTV-rtTA transgenic mice were mated to TetO-LacZ mice and bitransgenic mice were identified. Wild-type (non-transgenic) and bitransgenic mice were treated with doxycycline. Breast tissue from wild-type and bitransgenic mice was harvested after 48 hours of treatment with doxycycline, as was breast tissue from a bitransgenic littermate that had not been treated with doxycycline. RNA was prepared from these three tissue samples and steady-state levels of *rtTA* and *LacZ* mRNA expression were assessed by Northern hybridization (Fig. 4B). As expected, bitransgenic animals expressed *rtTA* at similar levels in the presence and absence of doxycycline, whereas the *LacZ* target mRNA was only expressed in bitransgenic animals treated with doxycycline. No *LacZ* mRNA was detected either in wild-type animals, or in untreated bitransgenic animals.

In order to quantitate the level of lacZ protein expression in the induced and uninduced states, beta-galactosidase assays were performed on protein extracts made from breast tissues harvested from wild-type, MTB, TZA and MTB/TZA mice (Figs. 4C and 5). As expected, abundant beta-galactosidase activity was present in extracts prepared from bitransgenic animals treated with doxycycline for 48 hours.

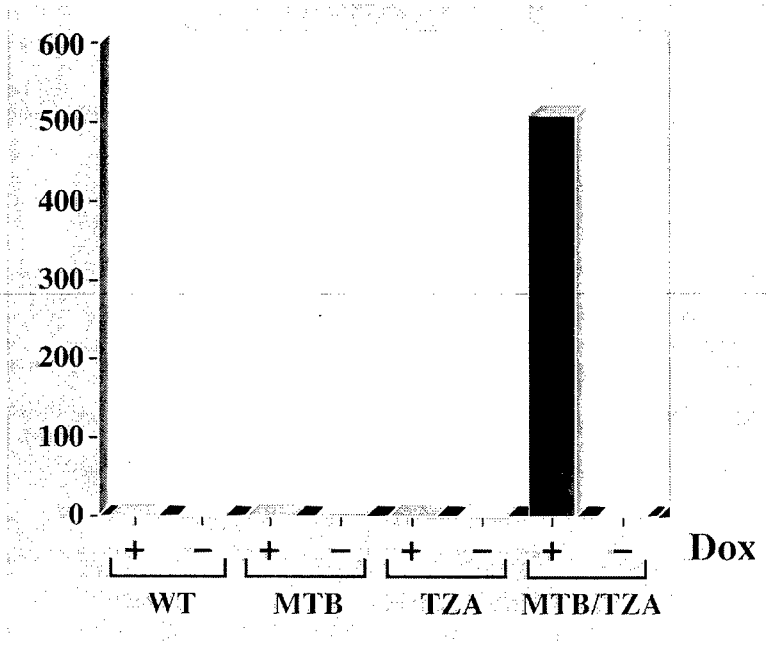

Fig. 5: Genotype-dependence for inducible overexpression of LacZ in bitransgenic mice

Impressively, no beta-galactosidase activity was detected in extracts prepared from breast tissue harvested from non-transgenic, MTB, or TZA mice in the presence or absence of treatment with doxycycline. In addition, no beta-galactosidase activity was detected in protein extracts prepared from untreated bitransgenic animals. Based on the lower limits of detection for this assay, we estimate that the minimum induction of transgene expression observed in this experiment is at least 700-fold, and that the actual level of induction may be 1 or 2 logs higher. Our estimates of the minimum level of induction are comparable to that observed in a related system making use of the tTA tetracycline-dependent repressor, rather than the rtTA tetracycline-dependent transactivator[28].

In order to further characterize this system, the time-course for transgene induction by doxycycline was determined (Fig. 6). Breast tissue from MTB/TZA bitransgenic mice treated with 2 mg/ml

doxycycline for the indicated periods of time was harvested and protein extracts prepared. MTB and TZA transgenic animals treated with doxycycline for 7 days were used as negative controls. Beta-galactosidase

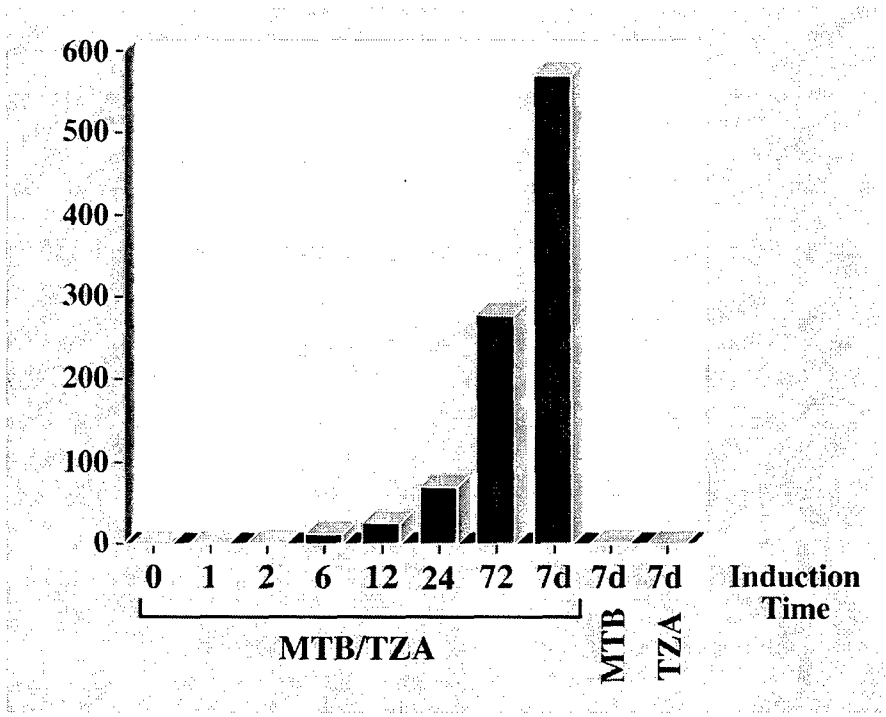

Fig. 6: Time-course of induction of LacZ expresssion in bitransgenic mice

assays revealed that transgene expression could be detected beginning at 6 hours following treatment with doxycyline. This initial increase represented at least a ten-fold induction of beta-galactosidase activity. Transgene expression continued to increase over the 7 day course of the experiment. No beta-galactosidase activity was detected in MTB or TZA control glands harvested from animals treated with doxycycline for 7 days.

Additional experiments performed to characterize this inducible model system included the determination of the doxycycline concentration-dependence of transgene induction (Fig. 7). MTB/TZA bitransgenic mice were treated for 72 hourse with doxycycline concentrations ranging from 0 to 8 mg/ml. Mammary tissue was subsequently harvested and protein extracts prepared. MTB and TZA transgenic animals treated with 8.0 mg/ml doxycycline for 72 hours were used as negative controls. Beta-galactosidase assays revealed that transgene expression could be detected in animals treated with as little as 0.03 mg/ml doxycycline. Treatment of bitransgenic animals with this dose of doxycycline resulted in a ten-fold induction of beta-galactosidase activity. Transgene expression increased as a function of doxycycline concentration and plateaued at 0.5 mg/ml. No beta-galactosidase activity was detected in MTB or TZA control glands harvested from animals treated with 8.0 mg/ml doxycycline.

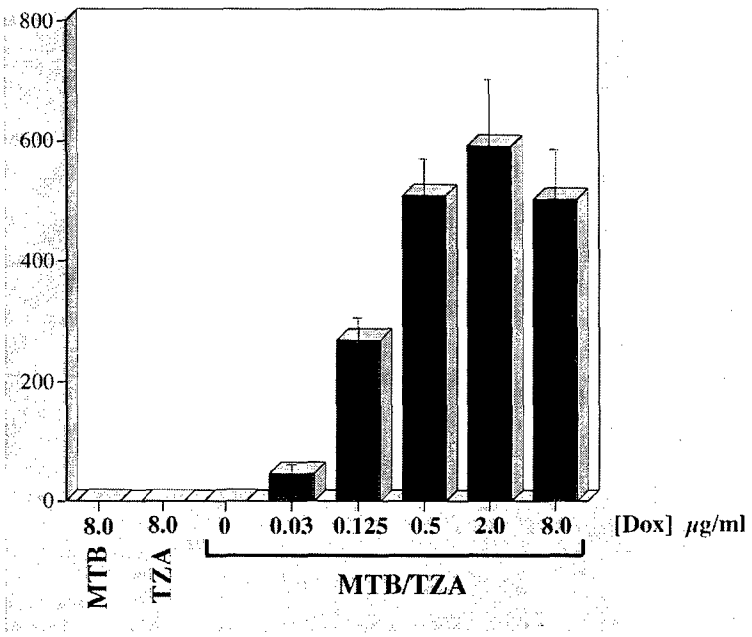

Fig. 7: Doxycycline concentration dependence of LacZ induction in bitransgenic mice

In order to confirm these results and to identify the cell types in the mammary gland in which inducible expression occurred, MMTV-rtTA transgenic mice were mated to TetO-LacZ mice and bitransgenic mice were identified. Bitransgenic mice were treated with doxycycline and breast tissue was harvested after 48 hours, as was breast tissue from a bitransgenic littermate that had not been treated with doxycycline. Both

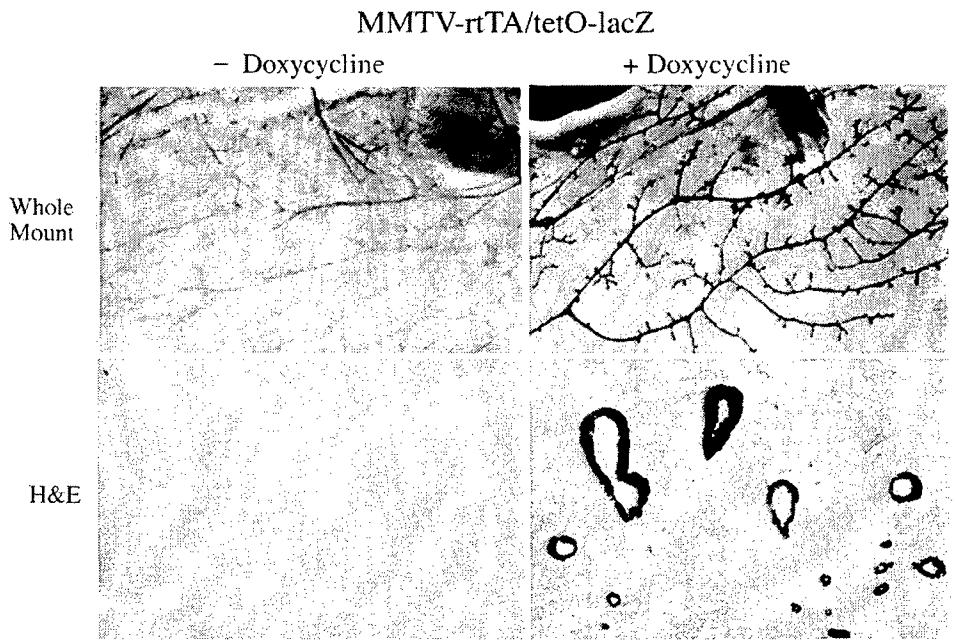

Fig. 8: LacZ expression in the mammary epithelium of bitransgenic mice

mammary gland whole-mounts and frozen sections from mammary glands embedded in OCT were stained *in situ* for lacZ activity (Fig. 8). Results from both whole-mounts and frozen sections demonstrated that inducible lacZ expression is confined to the mammary epithelial tree. Moreover, expression occurred at a high level and in a relatively homogeneous manner in all epithelial structures examined.

In aggregate, our results demonstrate that the MMTV-rtTA/TetO-transgene system that we have generated will permit the rapid and breast-specific induction of transgene expression at high levels, in combination with extremely low levels of expression in the uninduced state. Notably, this system permits the modulation of transgene expression levels both by varying the concentration of doxycycline and by varying the tetracycline derivative used. These properties are ideal for determining the effects of a potentially toxic transgene on specific stages of mammary gland development, and for obtaining levels of transgene expression that are in the physiological range.

In order to create transgenic lines of mice that express rtTA constitutively in all cell types in the mammary gland, including epithelial cells and stromal cells, the pHMG.rtTA transgene was injected into fertilized oocytes harvested from superovulated FVB female mice. Two founder mice, designated HTA and HTB, were identified that harbored the transgene in tail-derived DNA and that passed this transgene to offspring in a Mendelian fashion. In order to determine whether this transgenic system will permit inducible transgene expression in the breast in response to tetracycline analogs, HTA and HTB transgenic mice were mated to TetO-LacZ mice and bitransgenic mice were identified. Wild-type (non-transgenic) and bitransgenic mice were treated with doxycycline. Breast tissue from wild-type and bitransgenic mice was harvested after 48 hours of treatment with doxycycline, as was breast tissue from a bitransgenic littermate that had not been treated with doxycycline. Beta-galactosidase assays were performed on protein extracts prepared from each of these harvested breast tissues. No beta-galactosidase activity was detected in bitrasngenic HMG.rtTA/tetO-LacZ mammary tissue from animals induced with doxycycline. We conclude that the HTA and HTB lines of mice will not be suitable for expressing inducibly target transgenes in the mammary gland.

In order to create transgenic lines of mice that express rtTA in a tetracycline-dependent manner, purified DNA containing the pTetO.rtTA transgene was injected into fertilized oocytes harvested from superovulated FVB female mice. Two founder mice, designated TTB and TTE, were identified that harbored the transgene in tail-derived DNA and that passed this transgene to offspring in a Mendelian fashion. Northern hybridization analysis of tissues derived from progeny of the TTB and TTE lines of mice failed to reveal detectable levels of rtTA expression in the mammary glands of female virgin animals (not shown). Low levels of expression of rtTA were detected in TTB and TTE animals in the thymus, kidney and spleen. In order to confirm these results, the TetO.rtTA transgenic lines of mice, TTB and TTE, were mated to TetO-LacZ mice and bitransgenic tetO.rtTA/tetO-LacZ mice were identified. Wild-type and bitransgenic mice were treated with doxycycline. Breast tissue from wild-type and bitransgenic mice was harvested after 48 hours of treatment with doxycycline, as was breast tissue from a bitransgenic littermate that had not been treated with doxycycline. Beta-galactosidase assays were performed on protein extracts prepared from each of these harvested breast tissues. No beta-galactosidase activity was detected in bitrasngenic tetO.rtTA/tetO-LacZ mammary tissue from animals induced with doxycycline. We conclude that the TTB and TTE lines of mice will not be suitable for inducibly expressing target transgenes in the mammary gland.

Task 2: *Create and identify transgenic lines of mice expressing wild type and mutant forms of BRCA1 in a tetracycline-dependent manner.*

As described above, we have constructed a mammalian expression vector, pTetO.BRCA1, in which expression of *BRCA1* is driven by the tetO-containing promoter cassette. This constructs should permit the inducible expression of *BRCA1* in response to tetracycline in cells expressing the rtTA transcriptional

activator. Using an approach similar to that described above this construct, which also contains SV40 splicing and polyadenylation signals, has been used to generate transgenic mice in the FVB background. Initially, we identified two founder animals, TB1A and TB1B, that passed the pTetO-BRCA1 transgene to offspring in a Mendelian fashion. In order to generate more founder lines in order to increase the likelihood of identifying a tetO-BRCA1 line of transgenic animals that is able to inducibly express BRCA1 in response to doxycycline, we have reinjected purified pTetO.BRCA1 DNA into fetilized oocytes from superovulated FVB mice. This has resulted in the generation of an additional 7 founder animals that contain this transgene in their tail-derived DNA. These additional lines of mice have been bred to wild-type FVB mice in order to determine whether they pass this transgene to their offspring in a Mendelian fashion. In order to indentify lines of TB1 mice capable of inducibly expressing BRCA1, TB1 transgenic lines displaying Mendelian inheritance were mated to MTB transgenic mice, and the resulting MTB/TB1 bitransgenic mice were analyzed for BRCA1 transgene expression in the presence of doxycycline (Fig. 9).

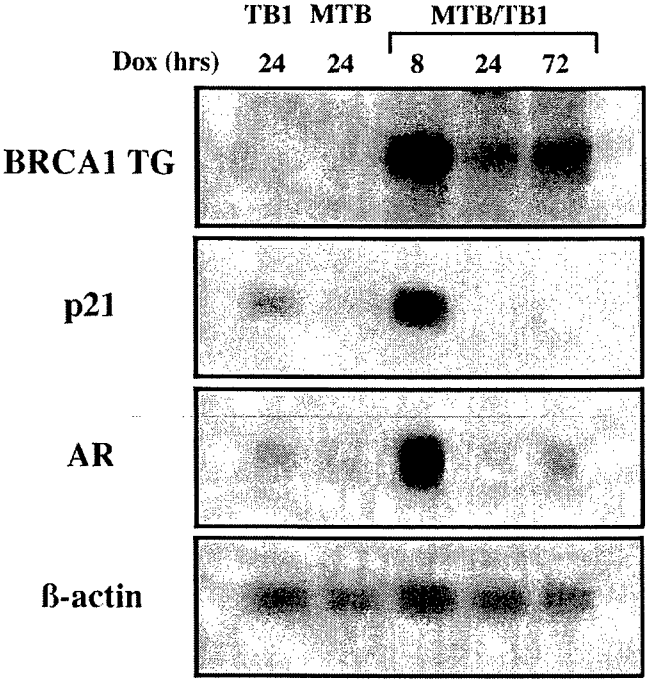

Fig. 9: Inducible BRCA1 expression in MTB/TB1 bitransgenic mice

This analysis resulted in the identification of two TB1 founder lines that inducibly express BRCA1 in the presence of an MTB transgene and in the presence of doxycycline induction. BRCA1 transgene induction was seen within 8 hours of induction. As expected, no induction was seen in TB1 or MTB transgenic animals treated with doxycycline. Lower levels of BRCA1 transgene expression were observed following either 24 hours or 72 hours of induction with doxycycline. It is not known whether this reflects animal to animal variation in inducibility, or whether high levels of BRCA1 induction result in apoptosis of expressing cells. In order to determine whether the BRCA1 expressed is functional, Northern blots were probed with cDNA probes for p21/WAF1/CIP1 and amphiregulin. Each of these genes has previously been reported to be upregulated by BRCA1. This analysis revealed that both p21 and amphiregulin are upregulated to high levels in MTB/TB1 bitransgenic mice treated with doxycycline for 8 hours. This analysis stronlgy suggests that we have succeeded in generating a bitransgenic system that is capable of inducibly expressing function BRCA1 protein in the murine mammary gland *in vivo*.

Task 3: *Create and identify transgenic lines of mice expressing BRCA1 antisense RNA in a tetracycline-dependent manner.*

Before creating transgenic mice expressing *BRCA1* antisense RNA in a tetracycline-dependent manner, we wished to determine whether the pTetO-BRCA1.AS construct generated above would be capable of down-regulating *BRCA1* expression. In order to accomplish this, the antisense constructs are first being tested for their ability to specifically reduce the expression of BRCA1 protein using the HC11 *in vitro* test system that we have generated. Achieving this goal requires the specific detection of mouse Brca1 protein. We have tested several commercially available antisera directed against mouse Brca1. In our hands, none of these antisera is capable of specifically detecting mouse Brca1. As a result, we have chosen to generate our own anti-mouse Brca1 antisera. GST fusion proteins from four regions of the mouse Brca1 protein have been generated and expressed in E. coli. GST fusion proteins have been cleaved, purified and each has been injected into two rabbits. The resulting antisera are being tested for their ability to specifically recognize the mouse Brca1 protein. Preliminary evidence indicates that we have generated at least one antisera that specifically recognizes mouse Brca1. Using this antisera, we will screen the above HC11 clones that inducibly express BRCA1 antisense in order to determine whether the antisense fragments selected are capable of down-regulating Brca1 at the protein level.

If these experiments are successful, a DNA fragment containing the pTetO-BRCA1.AS expression cassette will be injected into fertilized oocytes harvested from superovulated FVB female mice, using an approach similar to that described above. Founder mice will be identified that harbor the transgene in tail-derived DNA and that pass this transgene to offspring in a Mendelian fashion.

As a parallel approach to this problem, tetracycline-inducible expression vectors were created to conditionally express four different hammerhead ribozymes designed to specifically cleave the *Brca1* transcript. Hammerhead ribozymes are catalytic RNAs that efficiently cleave RNA and thereby down-regulate gene expression. Hammerhead ribozymes can cleave any RNA containing its 5'-UH-3' consensus sequence where U can be replaced by a C, and H=C, U or A. Hammerhead ribozymes effectively and selectively inhibit gene expression in bacteria, plants, cell culture and animals, and colocalization of the ribozyme with the target RNA is critical to the efficacy of the system. We have stably integrated pTetO-Ribo1, pTetO-Ribo2, pTetO-Ribo3, and pTetO-Ribo4 into HC11 cells. Each of the resulting cell lines has undetectable expression of the ribozyme in the uninduced state. These clones will now be tested to assess the ability of each ribozyme to inhibit *Brca1* expression. The efficacy of the ribozymes will be assessed by RNAP assays, and with antisera directed against murine Brca1. In order to assess the specificity of the ribozymes to the *Brca1* transcript, *Actin* and *Gapdh* levels will be examined. Also, a non-specific ribozyme was designed as a negative control. To test the efficacy of the ribozymes, clones will initially be screened after 24 hrs. exposure to doxycycline. In the event that no inhibition of *Brca1* expression is observed, clones will be screened for inhibition at later time points.

Task 4: *Breed transgenic lines of mice to create a bitransgenic line of mice expressing wild type and mutant forms of BRCA1 in the breast in a tetracycline-dependent manner.*

MMTV-rtTA, MTB, transgenic mice identified above have been mated to offspring of the TetO-BRCA1, TB1, transgenic lines described above. Bitransgenic offspring have been identified by PCR. The expression of BRCA1 has been induced in bitransgenic animals by treatment with doxycycline. Breast tissue has been harvested from treated and untreated bitransgenic animals as well as control non-transgenic animals. BRCA1 expression in the breast has been assayed by Northern hybridization. As

described above, our analysis has demonstrated that MTB/TB1 bitransgenic mice inducibly express function BRCA1 in the presence of doxycycline..

Task 5: *Breed transgenic lines of mice to create a bitransgenic line of mice expressing BRCA1 antisense in the breast in a tetracycline-dependent manner.*

The completion of this task is pending at the current time. Completion of this task will depend on the successful completion of Task 3 in which transgenic lines of mice are created that express BRCA1 antisense RNA in a tetracycline-dependent manner, and that downregulate the Brca1 protein when tested in HC11 cells..

Technical Objective III: Determine the effect of inducibly overexpressing BRCA1 in the mammary epithelium of transgenic mice during specific developmental stages.

Task 1: *Analyze the phenotype of inducibly overexpressing wild type BRCA1 in the mammary epithelium during specific developmental stages.*

Since we have now successfully generated bitransgenic lines of mice that express wild type forms of BRCA1 in the breast in a tetracycline-dependent manner, we are currently analyzing the effect of inducibly overexpressing BRCA1 in the mammary epithelium during specific developmental stages. These include: puberty, early pregnancy, late pregnancy, lactation and early postlactational regression. Glands of induced and uninduced animals will be analzed for proliferation, differentiation and apoptosis in order to determine the effect of BRCA1 expression.

Task 2: *Analyze the phenotype of inducibly overexpressing mutant forms of BRCA1 in the mammary epithelium during specific developmental stages.*

The completion of this task will depend on the successful completion of tasks described above in which bitransgenic lines of mice are generated that express mutant forms of BRCA1 in the breast in a tetracycline-dependent manner. Constructs permitting the inducible expression of mutant forms of BRCA1 are currently being generated and will be injected in the oocytes of superovulated FVB mice.

Technical Objective IV: Determine the effect of inducibly abolishing BRCA1 expression in the mammary epithelium of transgenic mice during specific developmental stages.

Task 1: *Analyze the phenotype of inducibly abolishing BRCA1 expression in the mammary epithelium during specific developmental stages.*

The completion of this task will depend on the successful completion of tasks described above in which bitransgenic lines of mice are generated that express BRCA1 antisense RNA in the breast in a tetracycline-dependent manner and that result in the specific reduction in BRCA1 protein in mammary epithelial cells.

KEY RESEARCH ACCOMPLISHMENTS

- Construction of plasmid vectors for expressing rtTA in the mammary epithelium in a tetracycline-dependent or tetracycline-independent manner.
- Construction of target vectors for expressing wild-type and mutant forms of BRCA1.
- Construction of target vectors for expressing BRCA1 antisense RNA.
- Construction of target vectors for expressing BRCA1 ribozymes.
- Generation of four transgenic lines of mice containing the TetO-rtTA, MMTV-rtTA, TetO-LacZ and TetO-BRCA1 expression cassettes.
- Demonstration that the MMTV-rtTA transgenic line of mice express the tetracycline-dependent reverse transcriptional activator, rtTA, at high levels, in a breast-specific manner, and in a mammary epithelial-specific manner.
- Demonstration using bitransgenic MMTV-rtTA/TetO-LacZ mice that this system permits the rapid induction of target gene expression to high levels in a breast-specific fashion in response to induction with tetracycline derivatives.
- Generation of bitransgenic mice that inducibly express BRCA1 throughout the mammary epithelium within hours following the administration of doxycycline.
- Demonstration that induction of BRCA1 in the mammary epithelium results in the induction of both p21/WAF1 and amphiregulin. This is the first demonstration of inducible expression of BRCA1 *in vivo*.

REPORTABLE OUTCOMES

Development of mammary epithelial cell lines, and transgenic mice, and anti-Brca1 antisera as described above.

Publications during the current contract year:

Chodosh LA. *BRCA1* and *BRCA2* expression in normal and neoplastic cells. *Journal of Mammary Gland Biology and Neoplasia* 3:389-402, 1998.

Chodosh LA, D'Cruz CM, Gardner HP, Ha SI, Marquis ST, Rajan JV, Stairs DB, Wang JY, and Wang M. Mammary gland development, reproductive history and breast cancer risk. *Cancer Research* 59:1765s-1772s, 1999.

CONCLUSIONS

A number of important milestones have been accomplished during the first three years of this project. We have constructed a variety of plasmid vectors for expressing rtTA in the mammary epithelium in a tetracycline-dependent or tetracycline-independent manner. We have also created target vectors for expressing wild-type and mutant forms of BRCA1, as well as target vectors for expressing BRCA1 antisense RNA. These vectors have been used to create four transgenic lines of mice containing the TetO-rtTA, MMTV-rtTA, TetO-LacZ and TetO-BRCA1 expression cassettes. Experiments to date demonstrate that the MMTV-rtTA transgenic line of mice express the tetracycline-dependent reverse transcriptional activator, rtTA, at high levels, in a breast-specific manner, and in a mammary epithelial-specific manner. The creation of bitransgenic MMTV-rtTA/TetO-LacZ mice has permitted the initial analysis of the utility of this inducible transgenic system. These results strongly suggest that this system is capable of inducing target gene expression to high levels in a breast-specific fashion in response to induction with tetracycline derivatives. In addition, this system demonstrates extremely low levels of basal expression. We have also succeeded in generating bitransgenic mice that inducibly express BRCA1 throughout the mammary epithelium within hours following the administration of doxycycline. In addition, we have demonstrated that induction of BRCA1 in the mammary epithelium results in the induction of both p21/WAF1 and amphiregulin. This is the first demonstration of inducible expression of BRCA1 *in vivo*. The studies described above demonstrate that we have made significant progress towards the completion of the specific aims of this project during the first three years. Our findings suggest that the experimental system that we have generated can be used to answer important scientific questions regarding the function of the breast cancer susceptibility gene, BRCA1, in the mammary gland.

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Expression of *BRCA1* and *BRCA2* in Normal and Neoplastic Cells

Lewis A. Chodosh[1,2]

Current evidence strongly supports a role for the breast cancer susceptibility genes, *BRCA1* and *BRCA2*, in both normal development and carcinogenesis. Valuable clues regarding the function of these genes have been garnered through studies of their patterns of expression. A central feature of the *in vivo* pattern of *BRCA1* and *BRCA2* expression is that each of these putative tumor suppressor genes is expressed at maximal levels in rapidly proliferating cells. This feature is consistent with *in vitro* observations that *BRCA1* and *BRCA2* are expressed in a cell cycle-dependent manner. This feature is also well illustrated during mammary gland development wherein the expression of *BRCA1* and *BRCA2* is induced in rapidly proliferating cellular compartments undergoing differentiation, such as terminal end buds during puberty and developing alveoli during pregnancy. Strikingly, the spatial and temporal patterns of *BRCA1* and *BRCA2* expression are virtually indistinguishable during embryonic development and in multiple adult tissues despite the fact that these genes are unrelated. These observations have contributed to the emerging hypothesis that these genes function in similar regulatory pathways.

KEY WORDS: *BRCA1; BRCA2*; gene expression; development.

INTRODUCTION

Breast cancer is the most common malignancy diagnosed among women in United States, and is the second leading cause of cancer mortality. Despite intensive efforts aimed at improving the early detection and treatment of breast cancer, mortality from this disease has only recently begun to decline. In this setting, strategies aimed at a more thorough understanding of the underlying biology of this disease are likely to be important. The markedly elevated risk of breast cancer observed in women carrying germline mutations in *BRCA1* and *BRCA2* strongly suggests that these genes play a critical role in the regulation of mammary epithelial cell growth. Moreover, the findings that the BRCA1 protein is rapidly phosphorylated following DNA damage, and that BRCA1 and BRCA2 each interact with the recombination repair protein Rad51, have implicated these genes in the cellular response to DNA damage (1). As such, studies of *BRCA1* and *BRCA2* function will likely provide insight into the mechanisms of growth control and DNA damage response in normal mammary epithelial cells, as well as serve as a foundation for understanding how the absence or mutation of these molecules promotes carcinogenesis.

Insights into gene function have frequently been gained by studying patterns of regulation during development and carcinogenesis. In particular, inferences made from the spatial and temporal patterns of *BRCA1* and *BRCA2* expression have provided the basis for several fundamental hypotheses regarding the function of these molecules. Perhaps most striking has been the observation that *BRCA1* and *BRCA2* expression patterns in a variety of tissues are virtually superimposable. These studies, when taken together with other similarities in

[1] Department of Molecular and Cellular Engineering and Department of Medicine, Division of Endocrinology, Diabetes and Metabolism, Stellar-Chance Laboratories, Room 309A, University of Pennsylvania School of Medicine, 422 Curie Boulevard, Philadelphia, Pennsylvania 19104–6100.

[2] To whom correspondence should be addressed. e-mail: chodosh@mail.med.upenn.edu

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the structure and mutational profile of these genes, have contributed to the growing realization that BRCA1 and BRCA2 are likely to function in similar pathways.

GENE STRUCTURE AND EXPRESSION

Although the *BRCA1* and *BRCA2* genes do not share any significant sequence homology, these genes are similar in several respects. Both *BRCA1* and *BRCA2* are poorly conserved among species. Both have a similar genomic structure with an unusually large exon 11, 3.4 kb for *BRCA1* and 5 kb for *BRCA2* (Fig. 1). The *BRCA1* gene is composed of 23 exons and is expressed as a 7.8 kb mRNA transcript (2,3). This transcript encodes a protein of 1863 amino acids with a predicted size of 206 kDa, though the protein migrates on denaturing polyacrylamide gels with an apparent molecular weight of 220 kDa (1,4–9). Both the genomic structure and organization of *BRCA1* are conserved between mouse and human (10). Murine *Brca1* is expressed as a 7.5 kb mRNA transcript that encodes a protein of 1812 amino acids (10–13). The murine protein has 58% amino acid identity (72% similarity) with the human protein, but contains domains with considerably higher degrees of conservation, including the amino-terminal RING finger and carboxy-terminal BRCT domain (10–13). Unlike human BRCA1, for which several excellent immunologic reagents now exist, few reagents have been described that specifically recognize the mouse Brca1 protein.

The *BRCA2* gene is composed of 27 exons and is expressed as an 11.5–12 kb mRNA transcript (14,15). This transcript encodes a protein of 3418 amino acids with an estimated molecular weight of 384 kDa (14,15). While this protein has been detected in mammalian cell extracts, its apparent molecular weight is difficult to estimate relative to known markers given its large size (16). Mouse *Brca2* is expressed as an 11–11.5 kb mRNA transcript that encodes a protein of 3329 amino acids (17–19). The murine protein has 59% amino acid identity (72% similarity) with the human protein (17,18,20). Rat *Brca2* encodes a protein of 3343 amino acids that is 84% identical to mouse and 58% identical to human BRCA2 (18). As with Brca1, there are several domains of the mouse and rat Brca2 proteins that are highly homologous to their human orthologue, including amino and carboxyl terminal domains as well as several of the BRC repeats in exon 11 (17,18,20).

EXPRESSION OF SPLICE VARIANTS

Analysis of the structure of several cDNA clones described in the initial cloning of *BRCA1* implied that multiple alternatively spliced forms of *BRCA1* exist (2). Several splice variants of *BRCA1* have in fact now been described, among which the *BRCA1*-Δ9,10 and *BRCA1*-Δ11b variants are the best characterized (21, 22). Importantly, the reading frame of these alternatively spliced forms is preserved, such that each would be predicted to contain the highly conserved N-terminal RING domain and the C-terminal BRCT and transcriptional activation domains. The *BRCA1*-Δ9,10 splice variant deletes the 41 amino acids encoded by exons 9 and 10 and has been shown to be expressed at levels comparable to that of full length *BRCA1* in a variety of normal tissues and cell lines (21,22) . A second major splice variant, *BRCA1*-Δ11b, retains only the first 118 nt of exon 11 (21,22). This variant is also expressed at levels comparable to that of full length *BRCA1* in normal tissues and is generated by an alternative splicing event utilizing a donor site present at

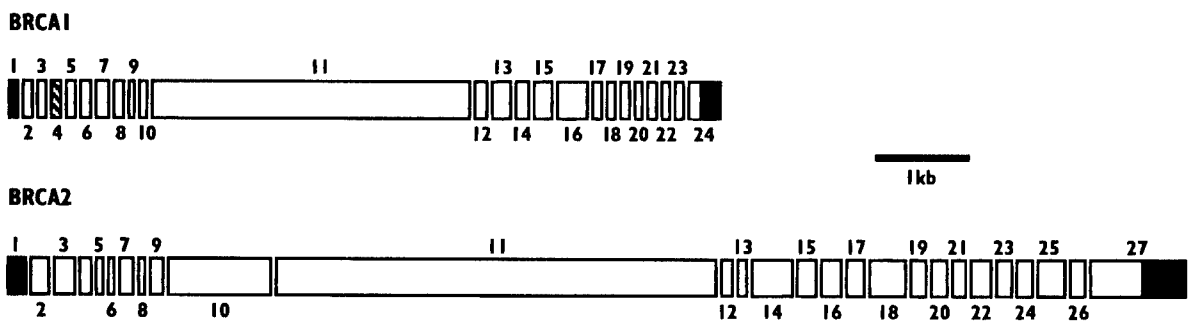

Fig. 1. Genomic Organization of *BRCA1* and *BRCA2*. Noncoding exons (shaded boxes) and coding exons (open boxes) of the human *BRCA1* and *BRCA2* genes are shown to scale. The alternate first exons, 1A and 1B, of *BRCA1* are not shown (see Figure 3). A nontranscribed region of *BRCA1* originally misidentified as exon 4 is indicated by crosshatching. The translational initiation codon for each gene is located in exon 2.

nucleotide positions 905–909 in exon 11. The *BRCA1*-Δ11b transcript encodes a 760 amino acid polypeptide with a predicted size of 85 kDa, though the protein migrates on denaturing polyacrylamide gels with an apparent molecular weight of 110 kDa (21). When transfected into mammalian cells, the protein encoded by the *BRCA1*-Δ11b transcript is cytoplasmic, consistent with the fact that the spliced region of exon 11 encompasses the nuclear localization signal (21). Intriguingly, although *BRCA1*-Δ11b is expressed at a level comparable to that of full-length *BRCA1* in several tissues, steady-state levels of the *BRCA1*-Δ11b message have been reported to be markedly reduced or absent in several breast and ovarian tumor cell lines (21). However, this reduced ratio of *BRCA1*-Δ11b:*BRCA1* transcripts is also observed in normal breast tissue as well as in breast tumors, suggesting that tissue-specific rather than tumor-specific splicing patterns may be operative (21). Several investigators have detected 4.4–4.6-kb mRNA species by Northern hybridization analysis of tissues and cell lines using non-exon 11-containing probes (22–24). These are likely to represent *BRCA1*-Δ11b transcripts. Moreover, this 4.4 kb message has been shown by one group to be associated with polysomes, consistent with its proposed translation (22). Exon 11b-negative mRNA transcripts are also found in the mouse during early embryogenesis as well as in embryonic stem cells (25). That this alternatively spliced form appears to have been conserved evolutionarily suggests that it may play an important physiological role.

An additional splice variant has been analyzed and shown to lack exon 11 in its entirety (nt 672–4095) (24). This variant is predicted to encode an 81 kDa protein. In vitro transcription and translation of a cDNA encoding this proposed splice variant generated a 97 kDa protein, consistent with the higher apparent as compared to calculated molecular weights of the full length BRCA1 and BRCA1-Δ11b proteins (24). Based on the observation that Northern hybridization of blots containing mRNA from human tissues using a *BRCA1*-Δ672–4095 DNA probe detects a 4.6 kb mRNA species, it has been proposed that the 4.6 kb band corresponds to *BRCA1*-Δ672–4095 (24). Although the existence of the *BRCA1*-Δ672–4095 isoform *in vivo* was confirmed by RT-PCR, as was the existence of an additional isoform, *BRCA1*-Δ789–4098, whether these transcripts are expressed at levels comparable to that of full length *BRCA1* or the *BRCA1*-Δ11b variant has not been determined (24). Given the similarity in size between the Δ672–4095, Δ789–4098 and Δ11b transcripts, as well as that of the polypeptides they are predicted to encode, the *in vivo* significance of the expression of the Δ672–4095 and Δ789–4098 isoforms remains uncertain (21,24). The existence of multiple tissue-specific splice variants of *BRCA1* has also been suggested based on the ability of Δexon11 probes to detect numerous mRNA species smaller than 7.8 kb on Northern blots containing mRNA from human tissues (24). Since few of these have been characterized in any detail or have been shown to be consistently present by independent investigators, it is unclear at present whether they represent alternatively spliced products or degradation products. Unlike *BRCA1*, multiple splice forms of *BRCA2* have yet to be described, though these clearly may exist.

EMBRYONIC EXPRESSION

The majority of current information regarding *BRCA1* and *BRCA2* expression during embryonic development has been obtained from studies conducted in mice. Northern hybridization and RNase protection analysis demonstrate *Brca1* mRNA expression in ES cells and embryos of FVB and C57BL/6 mice at E6.5–E7.5, consistent with the onset of developmental abnormalities in *Brca1-/-*mice (19,25,26). Similarly, *in situ* hybridization analysis reveals *Brca2* mRNA expression beginning at E7.5, coincident with the onset of developmental abnormalities in *Brca2-/-*mice. The expression of each gene is markedly up-regulated from E7.5 to E13.5 and subsequently down-regulated by E18.5 (10,17,19,27,28). *Brca1* and *Brca2* mRNA are expressed in all three germ layers and in virtually all cell types, with particularly high levels of expression noted in rapidly proliferating cell types undergoing differentiation such as the ventricular layer of the brain and germinal neuroblastic epithelium of the developing eye (10,19,27,29). In fact, spatial patterns of proliferation both during embryonic develoment and in adult tissues appear to be the strongest determinant of *Brca1* and *Brca2* mRNA expression patterns (19,29). Surprisingly, *in situ* hybridization analysis of serial sections of E18.5 embryos demonstrated essentially identical spatial patterns of expression of *Brca1* and *Brca2*, with high levels of expression observed in cellular compartments containing rapidly proliferating cells involved in differentiation, particularly developing neuroepithelium, toothbud, salivary gland, thymus, liver, lung, bowel and brown adipose tissue (Fig. 2) (19). *In situ* hybridization of E14–16 mouse embryos

Bright field
Brca2
Brca1
p21
Cephalic
Thoracic
Abdominal
mv
fv
po
oe
re
hf
lv
to
sg
tb
th
sc
bf
ht
di
sk
bl
bo
li
lu

demonstrated similar overlapping areas of *Brca1* and *Brca2* expression (19,20,28). The spatial coexpression of these genes contrasts markedly with that of the cell cycle regulatory protein $p21^{WAF1/CIP1}$ (Fig. 2). In addition to these initial studies demonstrating the striking spatial and temporal coexpression of *Brca1* and *Brca2* during embryonic development, *Rad51*, a protein that has been shown to interact directly or indirectly with both BRCA1 and BRCA2, is also spatially coexpressed with *Brca2* in the developing neuroepithelium of E8.5–E11.5 embryos (28,29,31).

EXPRESSION IN ADULT TISSUES

BRCA1 and *BRCA2* mRNA expression levels in adult tissues and cell lines are relatively low as assessed by *in situ* hybridization, Northern hybridization and RNase protection analysis (19,27). These assessments are supported by quantitative RNase protection and quantitative PCR analysis of *BRCA1* mRNA levels in cell lines, indicating that *BRCA1* mRNA is present in the HBL100 cell line at approximately 50 copies/cell, and at significantly lower levels in most other cell lines examined (21,32). This finding has important consequences for interpreting the physiological significance of experiments in which *BRCA1* expression is driven by relatively strong constitutive promoters since the resulting levels of *BRCA1* expression are almost undoubtedly supraphysiologic.

Similar to the pattern of expression in embryonic tissues, *BRCA1* mRNA can be detected in virtually all cell types and correlates most closely with proliferation (27,33). As originally reported, human *BRCA1* mRNA is expressed at highest levels in thymus and testis, with lower levels in breast and ovary (2). Similarly, the highest levels of *Brca1* mRNA expression in the FVB mouse are found in the testis, thymus and spleen with lower levels in breast, ovary and other tissues (2,10,13,27). Rat *Brca1* is also expressed at highest levels in the testis (34). *BRCA2* mRNA expression, like *BRCA1*, is found at highest levels in testis and thymus (14,19). Mouse *Brca2* mRNA is also expressed at highest levels in testis and thymus, with lower levels of expression in spleen, ovary, uterus, breast, and small intestine (17–19). Similarly, rat *Brca2* mRNA expression has been demonstrated at high levels in testis, with lower levels in thymus, spleen, mammary gland, ovary, prostate and heart. Thus, the tissue-specific patterns of *BRCA1* and *BRCA2* expression in adult tissues are remarkably similar in both human and mouse (14,19,20).

In situ hybridization analysis of adult tissues reveals that, as in the embryo, *Brca1* and *Brca2* are expressed in a wide variety of tissues and at highest levels in proliferating cellular compartments involved in differentiation, such as granulosa and thecal cells of developing ovarian follicles, endometrial glands in the uterus, the outer rim of the thymic cortex, and the basal epithelial cell layer in intestinal crypts of the small intestine and gastric glands in the stomach (19,27,29,35). The observation that *Brca1* and *Brca2* mRNA expression levels in the adult murine brain are low compared to other tissues is consistent with the positive correlation between *Brca1* and *Brca2* mRNA expression and cellular proliferation (10,18,19,27,36). In mouse testis, the highest levels of *Brca1* mRNA expression were detected in germ cells, specifically in pachytene spermatocytes and postmeiotic round spermatids, consistent with its suggested role in DNA repair and/or recombination (36). In other studies expression has been detected in both mitotic spermatogonia in addition to meiotic spermatocytes (29). Two groups have reported differences in the temporal patterns of *Brca1* mRNA expression relative to that of *Brca2* during spermatogenesis, though there is disagreement regarding the exact nature of these differences (19,29).

In light of the fact that much has been made of the relatively low level of evolutionary conservation between mouse and human BRCA proteins, it is interesting to note that the spatial pattern of *BRCA1* expression in primates is essentially identical to that described in the mouse (33). Specifically, *BRCA1* expression in

Fig. 2. *(opposite) In situ* hybridization analysis of *Brca1* and *Brca2* expression at E18.5. Bright field and dark field photomicrographs of serial frozen sections from the cephalic, thoracic or abdominal regions of day 18.5 mouse embryos hybridized to ^{35}S-labeled antisense or sense probes for *Brca2*, *Brca1*, or $p21^{WAF1/CIP1}$. A pair of arrows shows the anatomical limits of a crosssection through a loop of bowel, and illustrates the difference in hybridization pattern between *Brca2*, *Brca1*, and $p21^{WAF1/CIP1}$ in the bowel. **Key:** brown adipose tissue (bf); urinary bladder (bl); bowel (bo); diaphragm (di); fourth ventricle (fv); hair follicle (hf); heart (ht); liver (li); lung (lu); lateral ventricle of brain (lv); mesencephalic vesicle (mv); roof of midbrain (md); midgut (mg); olfactory epithelium (oe); pons (po); respiratory epithelium (re); spinal cord (sc); submandibular gland (sg); skin (sk); stomach (st); tooth bud (tb); thymus (th); tongue (to); ventricular layer of brain (vl).

cynomolgus monkeys is high in granulosa cells in maturing follicles of the ovary, in glandular and luminal epithelial cells of the endometrium, in the crypts of the intestine and gastric glands of the stomach, and in seminiferous tubules of the testis in primary spermatocytes and spermatids (33).

Finally, as observed during fetal development, *in situ* hybridization analysis of *Brca1* and *Brca2* mRNA expression in tissues of the adult mouse has revealed essentially identical patterns of expression in multiple tissues including breast, ovary, duodenum, uterus and thymus (19). Subtle differences in spatial expression have been noted in some tissues (19). Interestingly, these expression patterns are similar to those described for the mouse *Rad51* gene in adult tissues, although as yet the comparison has not been performed directly (37).

EXPRESSION IN THE MAMMARY GLAND

The spatial and temporal pattern of *Brca1* and *Brca2* expression in the mammary gland closely parallels that predicted from the patterns of expression observed in other tissues. Thus, in the breast as in other tissues both *Brca1* and *Brca2* are expressed in cellular compartments containing rapidly proliferating cells involved in differentiation, and both genes are expressed in a strikingly similar spatial and temporal pattern (10,19,27,29).

Brca1 and *Brca2* are predominantly expressed in the mammary epithelium throughout the postnatal development of the gland, with lower but clearly detectable levels in the stroma, particularly that immediately adjacent to the epithelial compartment (10,27). This pattern is also seen in the mammary glands of cynomolgus monkeys (33). Both *Brca1* and *Brca2* are expressed at significantly higher levels in the female breast compared to the male breast, presumably reflecting the greater amount of mammary epithelium present in the adult female. RNase protection analysis has demonstrated that *Brca1* and *Brca2* mRNA levels in the mammary glands of 2 week and 5 week-old immature virgin female mice are significantly higher than those found in the mammary glands of 10 week and 15 week-old mature virgin females (19,27). Consistent with this finding *in situ* hybridization performed on the mammary glands of immature mice revealed higher levels of *Brca1* and *Brca2* mRNA in terminal end buds than in adjacent ducts or in ducts from sexually mature animals (19,27,29). The potential physiological significance of the elevation in *Brca1* and *Brca2* mRNA levels at age 2 weeks, prior to the onset of puberty and the formation of terminal end buds, is interesting to contemplate. *Brca1* and *Brca2* mRNA levels are both markedly upregulated early in pregnancy, a period during which alveolar buds begin the process of rapid proliferation and differentiation to form mature, milk-producing alveoli (10,19,27,29). *In situ* hybridization demonstrates that this upregulation of *Brca1* and *Brca2* expression occurrs preferentially in developing alveoli as compared with adjacent epithelial ducts, consistent with patterns of proliferation (19,21). *Brca1* and *Brca2* expression levels decline late in pregnancy, reaching their nadir during lactation and early postlactational regression, though expression levels remain above background even during these stages of development (10,19,27,29). Interestingly, the mammary glands of parous mice that have undergone four weeks of postlactational regression have consistently been shown to express higher levels of *Brca1* and *Brca2* mRNA than the mammary glands of age-matched virgin controls (19). Whether this observation reflects changes in the differentiated state of the mammary epithelium, changes in the distribution of mammary cell types in various phases of the cell cycle, or other as yet unrecognized factors remains to be elucidated.

Despite these similarities in *Brca1* and *Brca2* expression patterns, discrete differences have been noted in the quantitative patterns of expression of these two genes during mammary gland development. First, the magnitude of the up-regulation in *Brca1* mRNA levels that occurs either during early pregnancy, or in ovariectomized animals treated with estradiol and progesterone to simulate early pregnancy, was significantly greater than that observed for *Brca2* (19). When coupled with the observation that the ratio of mRNA expression in the mammary glands of adult females relative to adult males was significantly greater for *Brca1* than for *Brca2*, these observations raise the possibility that pathways activated by androgens and/or ovarian hormones may have differential effects on the regulation of *Brca1* and *Brca2* expression.

Regarding potential relationships between normal mammary gland development and reproductive endocrine risk factors for breast cancer, the observation that *Brca1* and *Brca2* are each up-regulated in the breast during puberty and pregnancy, periods of development associated with both increases in cellular proliferation and increases in breast cancer risk in humans, raises the possibility that the induction of *Brca1* and *Brca2*

expression is a protective response to proliferation (27). The observation that *Rad51* is similarly up-regulated in proliferating cells is consistent with this hypothesis, and suggests that induction of these genes may be a consequence of the need to activate DNA damage repair pathways to respond to the genomic damage that accompanies rapid proliferative states. Finally, since few somatic mutations in *BRCA1* or *BRCA2* have been identified in sporadic breast cancers, the intriguing possibility remains that the function of these cancer susceptibility genes in the mammary gland may be restricted to specific developmental stages.

CELL CYCLE REGULATION OF EXPRESSION

The correlation between the proliferative status of tissues and *BRCA1* and *BRCA2* expression is also observed in cultured cells providing an opportunity for studies of the cell cycle-dependent expression of these genes (3,23,38–40). As in other contexts, the temporal pattern of *BRCA1* expression during the cell cycle is essentially indistinguishable from that of *BRCA2* (38–40).

BRCA1 and *BRCA2* mRNA expression are tightly regulated during mammary epithelial proliferation. *BRCA1* and *BRCA2* mRNA levels are high in exponentially growing cells and decrease in cells made quiescent by confluence or growth factor withdrawal (3, 38–40). Cells arrested in G0 or early G1 express low levels of *BRCA1* and *BRCA2* mRNA. After release from serum starvation and reentry into the cell cycle, *BRCA1* and *BRCA2* mRNA levels progressively increase during G1 reaching maxima at the G1/S transition (3,38,39). Peak expression of *BRCA1* and *BRCA2* occurs just prior to expression of histones *H2A* and *H2B*, and parallels expression of the S-phase-dependent marker, cyclin A (23,38,39). Interestingly, these expression patterns are similar to that described for the mouse *Rad51* gene (37). Synchronization of cells at the G1/S boundary further demonstrates that induction of *BRCA1* and *BRCA2* mRNA expression occurs prior to, and independent of, the onset of DNA synthesis (38,40). The cell cycle dependence of expression has been documented by synchronization via starvation or treatment with reversible cell cycle inhibitors, and by centrifugal elutriation (36). Importantly, changes in BRCA1 and BRCA2 protein levels parallel changes in *BRCA1* and *BRCA2* mRNA levels. Specifically, as assessed in synchronized cells, the amount of BRCA1 and BRCA2 protein increases during progression through G1, peaking during S phase and remaining elevated in G2/M as compared with cells in G0/G1 (5,7,16,41). This finding suggests that at least in this context steady-state mRNA levels are the major determinants of protein levels (5,7,16,37,41,42).

The cell cycle-dependent pattern of expression of *BRCA1* and *BRCA2* has been demonstrated in human primary mammary epithelial cells derived from reduction mammoplasties, in multiple immortalized, nontumorigenic mouse and human mammary epithelial cell lines, and in a variety of breast cancer-derived cell lines as well as in other cancer-derived cell lines (3,37,39). In contrast, it has been reported that MCF10A cells do not exhibit the cell cycle-dependent, and growth factor-dependent changes in BRCA1 protein expression seen in other cell lines (8). Whether these differences are due to characteristics of the MCF10A cell line used or the experimental conditions employed is unclear. Nevertheless, in aggregate these results clearly demonstrate that proliferative stimuli modulate the mRNA expression of *BRCA1* and *BRCA2*.

CELL CYCLE AND DNA DAMAGE-DEPENDENT REGULATION OF PHOSPHORYLATION

A key observation in studies of BRCA1 function was the observation that the mobility of BRCA1 in SDS polyacrylamide gels changes in a cell cycle-dependent manner, and that these changes in mobility reflect changes in the phosphorylation state of this protein (5,41,42). Changes in BRCA1 phosphorylation parallel cell cycle-dependent changes in protein level (7,41). Alterations in BRCA1 mobility in SDS polyacrylamide gels observed during the cell cycle are abolished by treatment of immunoprecipitates with phosphatase (5,7,41,42). Phosphatase treatment of BRCA1 isolated from G0/G1 enriched cells results in an increased electrophoretic mobility, suggesting that BRCA1 is partially phosphorylated even during G0/G1 (7). BRCA1 undergoes hyperphosphorylation during late G1 and S phases of the cell cycle and is transiently dephosphorylated early after M phase as assessed by imposing a reversible cell cycle block (7,41,42). Similar results are seen in untreated cells separated by centrifugal elutriation (7). The kinase(s)

responsible for the cell cycle dependent changes in BRCA1 phosphorylation is presently unknown.

BRCA1 has subsequently been shown to be a serine phosphoprotein (5,7). Two-dimensional tryptic peptide analysis of BRCA1 isolated from HeLa cells and from BRCA1 overexpressing 293T cells has revealed that BRCA1 is phosphorylated predominantly on serine and weakly on threonine (7). A low degree of phosphorylation has been detected on tyrosine by one group using phosphoamino acid analysis (6). Conflicting results have been obtained by other groups by immunoblotting with anti-phosphotyrosine antibodies (42,43).

In addition to cell cycle-dependent changes in BRCA1 phosphorylation, treatment of S-phase cells with a variety of DNA damaging agents, including ultraviolet radiation (UV), hydroxyurea, gamma radiation, mitomycin C, and hydrogen peroxide leads to phosphorylation of BRCA1 within approximately 20 minutes (41,42). Phosphorylation of BRCA1 in G1 phase cells does not occur following exposure to hydroxyurea, mitomycin C or low-dose UV-treated cells, although higher levels of UV irradiation are able to shift the G1 form of BRCA1 (41). Together these observations strongly suggest a role for BRCA1 in sensing or responding to DNA damage. This hypothesis is further supported by the physical association of both BRCA1 and BRCA2 with Rad51. In aggregate, current evidence favors the existence of a large multiprotein complex in the nucleus involved in the of sensing and/or response to DNA damage (1).

HORMONAL REGULATION OF EXPRESSION

It is likely that the effects of hormones on *BRCA1* and *BRCA2* expression can be best understood as a consequence of the cell cycle-dependence of *BRCA1* and *BRCA2* expression. Initial studies of the *in vivo* pattern of *Brca1* gene expression were interpreted as reflecting patterns or proliferation and differentiation rather than direct consequences of hormone exposure (10,21). For instance, the upregulation of *Brca1* and *Brca2* expression that occurs in the mammary gland in response to either pregnancy or treatment with estradiol and progesterone could mean either that the expression of these genes is directly regulated by steroid hormones, or that the induction of *Brca1* and *Brca2* expression is an indirect consequence of the rapid proliferation and differentiation of the mammary epithelium that occurs in response to these hormones. The observation that *Brca1* expression is markedly elevated in virtually all rapidly proliferating cellular populations regardless of whether they are hormonally-regulated was taken as support for the hypothesis that *Brca1* mRNA expression in the mammary gland reflects cellular proliferation induced by hormonal stimulation, rather than a direct effect of these hormones on gene expression *per se* (27).

Subsequent *in vivo* and *in vitro* studies have borne out this hypothesis for both *Brca1* and *Brca2*. In particular, *Brca1* and *Brca2* have been shown to be expressed at highest levels in granulosa and thecal cells of the small and medium ovarian follicles that grow independently of hormonal stimulation (29,35). Impressively, *Brca1* and *Brca2* continue to be expressed at high levels in this class of follicles even in the absence of hormonal stimulation, as shown by studies in both hypophysectiomized and estrogen-receptor-deficient mice (29,35). Consistent with this idea, *Brca1* and *Brca2* expression uniformly correlated with S-phase proliferating cell nuclear antigen expression (29,35).

Similarly, *in vitro* studies have demonstrated that treatment of estrogen receptor-positive mammary epithelial cell lines with estradiol stimulates proliferation of these cell lines and results in a coordinate increase in *BRCA1* and *BRCA2* mRNA levels (23,44,45). These estradiol-induced increases in *BRCA1* and *BRCA2* expression occur in parallel with the increases in S-phase-dependent markers such as cyclin A, rather than in parallel with classical estrogen-responsive genes such as pS2 (23). In addition, other hormones and growth factors that stimulate mitogenesis have a similar effect, including insulin like growth factor-1, epidermal growth factor, and progesterone (23,45). Conversely, estrogen depletion results in a reduction in *BRCA1* mRNA and protein levels in estrogen receptor-positive cell lines, as does treatment with agents such as TGF-β that inhibit mammary epithelial proliferation (3). Moreover, induction of *BRCA1*, but not pS2, is blocked by cycloheximide indicating that the estradiol-induced upregulation of *BRCA1* and *BRCA2* expression requires *de novo* protein synthesis and is therefore indirect (44,45). These observations strongly suggest that the ability of hormones to regulate *BRCA1* and *BRCA2* gene expression in specific cell types is primarily due to the ability of these agents to modulate proliferation in these cells, rather than to specific and direct effects of hormones on *BRCA1* and *BRCA2* expression *per se*.

REGULATION OF *BRCA1* AND *BRCA2* EXPRESSION IN RESPONSE TO OTHER STIMULI

Despite the strong correlation between *Brca1* and *Brca2* expression and proliferative status, an increasing number of instances have been described in which the expression of these genes appears to be influenced by factors other than proliferation. For instance, analysis of *Brca1* and *Brca2* mRNA levels in HC11 mammary epithelial cells reveals coordinate up-regulation in postconfluent cells treated with insulin and glucocorticoids (39). Steady-state levels of *Brca1* and *Brca2* mRNA expression in this context increase progressively over the course of several days to levels as high as those found in actively proliferating cells, despite the fact that rates of proliferation in these cells remain low and relatively constant. This up-regulation requires a serum factor that is removed by charcoal stripping (39).

Similarly, other investigators have argued that the dependence of *Brca1* and *Brca2* expression on proliferation is also abrogated in selected other tissues, such as in the testis and in differentiated neurons in the brain (29). Interestingly, *BRCA1* mRNA and protein expression have been reported to be transiently elevated in SKOV-3 cells following treatment with cis-diamminedichloroplatinum(II) (CDDP), stably elevated in A2780 cells chronically exposed to adriamycin or cisplatin, and stably elevated in CDDP-resistant variants of MCF-7 cells and SKOV-3 ovarian carcinoma cells (42,46). Confirmation that these increases in *BRCA1* expression are genuinely independent of proliferation will ultimately require elucidation of the pathways responsible for these effects. Nevertheless, studies of other tumor suppressor genes would suggest that *BRCA1* and *BRCA2* expression are likely to be controlled in a complex manner and to respond to diverse stimuli.

EXPRESSION IN BREAST CANCER

As expected on the basis of the widespread distribution of *BRCA1* expression *in vivo*, this gene is expressed in a wide variety of tumor cell types. It has been reported that in sporadic breast cancer *BRCA1* mRNA levels decrease during the transition from carcinoma *in situ* to invasive cancer (47). Relative to normal mammary epithelium, *BRCA1* mRNA expression was found to be increased in ductal carcinoma *in situ* and decreased in invasive cancer. Of four informative sporadic breast cancer cases examined, decreased *BRCA1* expression appeared to involve both *BRCA1* alleles in three cases (47). Whether the fourth case represented down-regulation of one allele by mutation, or loss of heterozygosity was not reported (41). If confirmed, it is unclear whether this downregulation of *BRCA1* expression in invasive cancer is more likely to represent selection for decreased BRCA1 activity in the course of tumor progression (i.e. a causal event) or a secondary effect occurring as a consequence of changes in upstream regulatory pathways controlling *BRCA1* expression. In this regard, it is important to note that the possibility of confounding effects of differences in proliferation rates on *BRCA1* expression were not assessed in this study, as it was performed prior to the recognition of the importance of this variable.

It has been somewhat difficult to reconcile the observed decrease in *BRCA1* expression in invasive breast cancers with observations that levels of *Brca1* mRNA expression in mammary epithelial cells *in vitro* are similar in transformed and non transformed cells (10,27,39). Furthermore, *Brca1* mRNA expression levels have been shown to be similar in normal rat mammary glands compared with mammary tumors induced either by the carcinogens DMBA or NMU, or by the activated-*neu* or activated-*ras* oncogenes (34). Finally, quantitative mRNA *in situ* hybridization performed on archival tumor specimens from patients with characterized *BRCA1* mutations and from patients with sporadic breast cancers revealed that, while *BRCA1* mRNA levels were invariably low in tumors from *BRCA1* mutations carriers relative to surrounding normal epithelium, only one third of sporadic tumors showed decreased *BRCA1* mRNA levels relative to surrounding normal breast epithelium (48). Clearly, additional data are needed to fully understand these findings.

The observation that somatic mutations in the coding regions of *BRCA1* and *BRCA2* are extremely rare in sporadic tumors, as well as the observation that *BRCA1* mRNA expression may be reduced in invasive breast tumors, has prompted a search for either regulatory mutations in the promoter regions of these genes or epigenetic mechanisms such as methylation that might account for their loss of activity in tumors in the absence of somatic mutations. The presence of regulatory mutations in familial breast cancer families was previously inferred from the existence of individuals heterozygous for a series of polymorphisms in the *BRCA1* gene, while mRNA isolated from these indi-

viduals appeared homozygous at these loci (2). Such inherited regulatory mutations could potentially cause reduced transcription, decreased mRNA stability, or aberrant splicing of the *BRCA1* transcript (2). Similar loss of transcription from one *BRCA1* allele has been reported in other families (49,50). Interestingly, the mutation in at least one of these families has been identified as a 14 kb deletion that removes exons 1a, 1b and 2 of *BRCA1*, thereby deleting both transcriptional initiation sites (49). Unequal crossover between Alu repeats appeared to be the most likely cause for this deletion, a significant observation given the fact that the *BRCA1* genomic sequence contains many such repeats. It has been proposed that 10–30% of germline mutations in *BRCA1* may be large deletions, and that up to half of these may affect the promoter region (51).

The observation that methylation of tumor suppressor genes such as *MTS1*, *RB1* and *VHL* has been implicated in decreased expression of these genes in cancer, suggests that epigenetic events may also play a role in sporadic breast cancer. Both the *BRCA1* and *BRCA2* genes are preceded by CpG islands (52). In contrast to most of the genome in which CpG dinucleotides are underrepresented and are constitutively methylated, CpG islands are typically unmethylated and represent potential sites for gene regulation via DNA methylation. The *BRCA1* CpG island extends over 1200 bp and includes exons 1a and 1b of *BRCA1* as well as its associated promoter region (53). In one study, the *BRCA1* promoter region in two of seven sporadic breast carcinomas was found to be hypermethylated (53). Hypermethylation was not detected in normal breast tissue samples or in samples of peripheral blood mononuclear cells. In a related study, an independent group identified CpG methylation in two of six breast carcinomas and two of five ovarian carcinomas, but not in normal tissues (54). The significance of these observations for the process of carcinogenesis is unknown. At present, compelling evidence for methylation in the *BRCA2* promoter region in breast or ovarian cancer cell lines is lacking (55).

PROMOTER ANALYSIS

BRCA1 transcription initiates from either of two sites separated by 277 bp and is under the control of two promoters that generate two distinct transcripts, alpha and beta, containing the alternatively spliced first exons, 1A and 1B (Fig. 3) (52,56,57). While both transcripts are expressed in most tissues and cell lines that have been examined, the alpha promoter appears to be the more potent of the two (54). Both the *BRCA1* alpha and beta promoters are TATA-less, although each contains GC-rich regions and has motifs similar to initiator elements as well as putative binding sites for the transcription factor, Sp1 (52,56,58). A role for a CCAAT box located in the intergenic region has been suggested based on transient transfection experiments (57). A variety of putative binding sites for other known transcription factors have been identified based on computer analysis although few have been directly tested. Neither promoter contains a classical estrogen response element. However, modest 1.5–2-fold increases in transcriptional activity in response to estradiol have been demonstrated in MCF-7 cells for fragments of the alpha and beta promoters containing either an AP1 site or an alternative estrogen response element (57). The failure of other investigators to detect a response to estrogen stimulation using genomic fragments near the 5′ end of the *BRCA1* gene may be due to differences in the genomic regions contained within these respective constructs (45).

In the human, a 30 kb tandem duplication of the genomic region containing the 5′ end of the *BRCA1* gene and a gene referred to as *NBR2* has been delineated (59) Fig. 3. The 5′ end of *NBR2* was initially believed to be a pseudocopy of *NBR1*, originally referred to as *1A1–3B*, which is itself located in a head-to-head configuration adjacent to a pseudocopy of the 5′ end of *BRCA1* that encompasses copies of exons 1a, 1b and 2 (59). Thus *BRCA1* is located in a head-to-head configuration with *NBR2*, while *NBR1* lies in a head-to-head configuration with a pseudocopy of the 5′ end of *BRCA1* (52,59,60). The transcription start sites of *BRCA1* and the adjacently located gene, *NBR2*, are only 218 bp apart. Promoter alpha is shared with the adjacent *NBR2* gene and is bi-directional (57). This complex arrangement raises the possibility that homology-mediated genetic rearrangements may lead to regulatory mutations in this gene that might not be detected by methods typically employed to search for *BRCA1* mutations (60).

The genomic organization of the 5′-flanking region of mouse *Brca1* gene is markedly different from that found in humans (61). The 30 kb genomic DNA fragment found in humans containing the *NRB2* gene and a pseudocopy of the 5′ end of the *BRCA1* gene is absent in mice (59). Moreover, alternative exon 1B of the human *BRCA1* transcript is not found in the mouse (57). These observations suggest that the mouse *Brca1* gene is unlikely to be regulated by two promot-

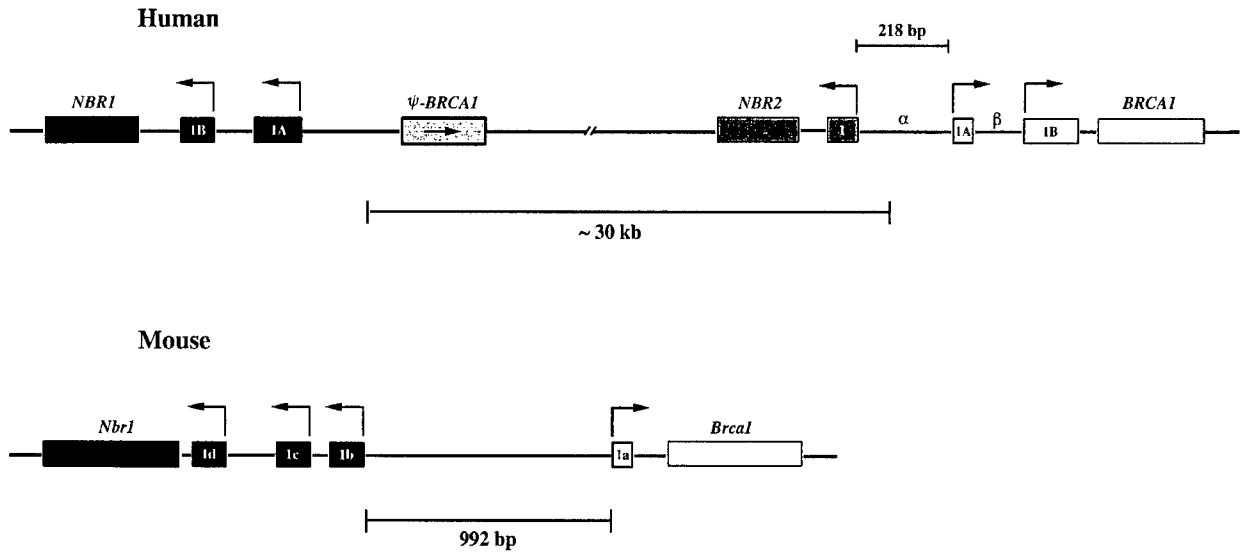

Fig. 3. Genomic structure of *BRCA1* locus and promoter region. Key: Open boxes, *BRCA1*; solid boxes, *NBR1*, light-shaded box, *BRCA1* pseudogene (ψ-*BRCA1*); dark-shaded box, *NBR2*. Alternate first exons of *BRCA1* and *NBR1* genes are shown. Rectangular arrows indicate transcription initiation sites and the direction of transcription. An internal arrow denotes the orientation of the BRCA1 pseudogene. The two promoter regions of human *BRCA1* are indicated as α and β. The diagram is not drawn to scale. Distances between the transcriptional initiation sites of *BRCA1* and *NBR2*, and between those of *Brca1* and *Nbr1* are indicated, as is the 30 kb duplicated genomic region of the human locus. Modified based on refs 56–61.

ers (57). While it has been suggested that the marked differences in the promoter regions of mouse and human *BRCA1* imply that the temporal and spatial pattern of expression of this gene may differ between the two species, it is clear that the temporal pattern of *BRCA1* expression during the cell cycle is indistinguishable for the mouse and human gene, and that the spatial pattern of *BRCA1* expression in multiple adult tissues is strikingly similar in mice and primates.

CONCLUSIONS AND FUTURE DIRECTIONS

Studies of the expression pattern of *BRCA1* and *BRCA2* have revealed multiple clues regarding the likely functions of these proteins. The best index of expression for these genes in a given population of cells is their proliferative status. As a result, any studies comparing expression levels of *BRCA1* or *BRCA2* between different tissues, cellular populations or developmental states must control for effects of proliferation on expression levels. Similarly, as a result of the finding that *BRCA1* and *BRCA2* expression levels are relatively low, caution must be used in interpreting studies in which these genes are massively overexpressed.

Notably, despite the relatively low extent of evolutionary conservation of the coding sequence and promoter regions of these genes, the patterns of regulated expression observed in the mouse, rat, monkey and human are nearly identical. These findings strongly argue that the evolutionary functions of these genes have been highly conserved. It will be of particular interest to determine the elements in the *BRCA1* and *BRCA2* promoters responsible for their cell cycle-dependent patterns of expression, and to compare the manner in which this regulation is achieved both between these different genes and for the same gene among different species. While it currently appears that the effects of hormones on *BRCA1* and *BRCA2* expression are primarily a consequence of their effects on proliferation and differentiation, further promoter analysis may be required to resolve this issue definitively.

One of the most striking findings to emerge from these studies is that *Brca1* and *Brca2* are expressed in similar tissue-specific patterns, at similar levels in a given tissue, and in similar cellular compartments within each tissue. In fact, during fetal development and in multiple adult tissues, the spatial and temporal patterns of *Brca1* and *Brca2* expression are virtually indistinguishable. This similarity is particularly evi-

dent during postnatal mammary gland development in the mouse, as each of these genes is up-regulated during puberty and pregnancy. A potential basis for this similarity is provided by the observation that *Brca1* and *Brca2* expression are coordinately regulated in proliferating and differentiating mammary epithelial cells *in vitro*. The remarkable extent to which *Brca1* and *Brca2* expression are coordinately regulated indicates that these genes are induced by similar stimuli, and suggests that they may function in overlapping pathways. Interestingly, similar overlapping expression patterns may be seen among subunits of heteromeric proteins. Whether or not the BRCA1 and BRCA2 proteins physically interact is currently a topic of intense investigation.

In contrast to recent advances in understanding *Brca1* and *Brca2* function in embryonic cells, relatively little is known about the function of these molecules in mammary epithelial cells. This deficiency is particularly relevant given that breast cancer is the predominant phenotype associated with mutation of these genes. For instance, the fact that germline *BRCA1* and *BRCA2* mutations specifically predispose carriers to breast cancer may relate to mammary-specific functions of these molecules. Moreover, there is as yet no explanation for the observation that *BRCA1* and *BRCA2* mutations appear to cause breast cancer only when present in the germline, since somatic mutations in these genes are not found in sporadic breast cancers. This finding may indicate that these molecules function in specific stages of mammary gland development, as might be predicted from their tightly regulated expression during mammary gland development. It should also be noted that *Brca1* and *Brca2* may function differently in embryonic versus adult cells. These findings, when considered with the possiblity that *BRCA1* and *BRCA2* may have mammary epithelial-specific functions, strongly argue that a full understanding of the role played by these genes in breast cancer susceptibility will require that their functions be studied directly in the mammary epithelium.

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Mammary Gland Development, Reproductive History, and Breast Cancer Risk[1]

Lewis A. Chodosh,[2] Celina M. D'Cruz, Heather Perry Gardner, Seung I. Ha, Sandra T. Marquis, Jayant V. Rajan, Douglas B. Stairs, James Y. Wang, and Man Wang

Departments of Molecular and Cellular Engineering [L. A. C., C. M. D., H. P. G., S. I. H., S. T. M., J. V. R., D. B. S., J. Y. W., M. W.], and Department of Medicine, Division of Endocrinology, Diabetes and Metabolism [L. A. C.], Stellar-Chance Laboratories, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6100

Abstract

The observation that normal pathways of differentiation and development are invariably altered during the process of carcinogenesis implies an intrinsic relationship between these processes. This relationship is particularly evident in the breast, as exemplified by the existence of endocrine risk factors for breast cancer that are related to the timing of normal developmental events. Understanding the mechanisms by which normal developmental events alter breast cancer risk is a central focus of our laboratory. Herein, we describe three approaches being taken in our laboratory toward defining the molecular basis of this relationship. These include: determining the roles played by the tumor suppressor genes, *BRCA1* and *BRCA2,* in the normal differentiation and development of the breast; studying the function of three novel protein kinases identified in our laboratory in mammary epithelial development; and defining the molecular and cellular changes that occur in the breast as a result of reproductive events known to influence breast cancer risk.

Introduction

A basic tenet emerging from studies in cancer biology is that normal pathways of differentiation and development are inevitably disrupted during the process of carcinogenesis. This implies an intrinsic relationship between these processes. The existence of endocrine risk factors for breast cancer that are related to the timing of normal developmental events such as menarche, menopause, and age at first full-term pregnancy epitomizes this relationship. The recognition that breast cancer risk is determined in part by the same reproductive endocrine events that drive mammary gland development argues that mammary gland development and mammary carcinogenesis are fundamentally related.

One of the most intriguing examples of this principle is the observation that women who undergo their first full-term pregnancy early in life (*i.e.*, early parity) have a significantly reduced lifetime risk of breast cancer (1). The magnitude of this parity-induced protection against breast cancer is similar in many countries and ethnic groups, regardless of endemic incidence. This suggests that protection results from an intrinsic effect of parity on the biology of the breast rather than from extrinsic factors specific to a particular environmental, genetic, or socioeconomic setting. This conclusion is bolstered by the observation that rats that have previously undergone a full-term pregnancy are resistant to the induction of breast cancer by administration of the carcinogen DMBA,[3] as compared to age-matched nulliparous controls (2, 3). Therefore, both human epidemiology and animal model systems support the conclusion that an early first full-term pregnancy results in a permanent change in the breast, either directly or indirectly, that confers a decreased risk for the subsequent development of breast cancer. Although this effect has been hypothesized to result from the impact of terminal differentiation on the susceptibility of the mammary epithelium to carcinogenesis, the molecular and cellular basis for this phenomenon is unknown.

A second illustration of this principle comes from the observation that breast cancer risk attributable to exposure to ionizing radiation is a function of age at the time of exposure. Specifically, studies of women who received mantle irradiation for Hodgkin's disease or who underwent repeated fluoroscopy in the course of treatment for tuberculosis have demonstrated that breast cancer risk is significantly greater in women who were exposed to ionizing radiation during adolescence as compared to women exposed at later ages (4, 5). Analogously, nulliparous rats fed DMBA are more likely to develop breast cancer if they are exposed during puberty rather than as mature adults (6). Interestingly, epidemiological studies suggest that the increased susceptibility of the immature human breast to early events in carcinogenesis may occur prior to as well as during puberty. Studies of survivors from Hiroshima and Nagasaki indicate that the greatest increase in breast cancer risk occurred in women who were less than 10 years old at the time of exposure (7). The observed increase in breast cancer incidence in women irradiated during the first year of life for presumed thymic enlargement is perhaps an even more impressive illustration of this principle, given the rudimentary state of the mammary gland at this age (8). Together, these studies suggest that the susceptibility of the mammary gland to carcinogenesis is related to the gland's developmental state at the time of exposure to mutagenic agents and that the immature breast is particularly susceptible to early events in carcinogenesis.

Understanding the molecular and cellular mechanisms by which normal developmental events alter breast cancer risk is a central goal of our laboratory. We believe that achieving this goal requires a more complete understanding of the manner in which hormones and reproductive history alter subpopulations of epithelial cell types present in the breast and of the roles played by key regulatory molecules in these processes. Toward this end, we are currently focusing on: (*a*) determining the roles played by the tumor suppressor genes, *BRCA1* and *BRCA2,* in the normal differentiation and development of the breast; (*b*) studying the function of three novel protein kinases identified in our laboratory in mammary epithelial development and carcinogenesis; and (*c*) defining the molecular and cellular changes that occur in the breast as a result of reproductive events known to influence breast cancer risk.

Tumor Suppressor Genes: *BRCA1* and *BRCA2*

The epidemiological relationship between development and carcinogenesis is illustrated on a molecular and mechanistic level by the existence and function of tumor suppressor genes such as *p53*, the Wilms' tumor gene (*WT1*), and the retinoblastoma susceptibility gene (*RB*). Germ-line mutations in these genes are associated with inherited cancer predisposition syndromes (9). The cloning and analysis of several tumor suppressor genes has revealed that they frequently

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[2] To whom requests for reprints should be addressed, at Department of Molecular and Cellular Engineering, Stellar-Chance Laboratories, Room 309A, University of Pennsylvania School of Medicine, 422 Curie Boulevard, Philadelphia, PA 19104-6100. Phone: (215) 898-1321; Fax: (215) 573-8590; E-mail: chodosh@mail.med.upenn.edu.

[3] The abbreviations used are: DMBA, 7,12-dimethylbenz[*a*]anthracene; EGFR, epidermal growth factor receptor.

encode proteins that act as negative regulators of cell proliferation, exert cell cycle checkpoint control function, or maintain genome integrity (10, 11). In addition, the targeted deletion of these genes in mice frequently results not only in increased susceptibility to cancer but also in abnormalities in proliferation, apoptosis, differentiation, and development (10, 12). As such, one approach to elucidating the relationship between mammary gland development and carcinogenesis is to determine the function of tumor suppressor genes known to be involved in the pathogenesis of breast cancer.

Genetic analysis of families in which multiple individuals have developed breast cancer suggests that 5–10% of breast cancer cases result from the inheritance of germ-line mutations in autosomal dominant susceptibility genes (13, 14). Over the past 4 years, several of these breast cancer susceptibility genes have been isolated by positional cloning, including *BRCA1* and *BRCA2* (15–19). Tumors arising in patients with germ-line mutations in either *BRCA1* or *BRCA2* typically display loss of the corresponding wild-type allele, suggesting that *BRCA1* and *BRCA2* are tumor suppressor genes (20–22). Interestingly, *BRCA1* and *BRCA2* mutations have not been identified in sporadic breast cancers, despite the fact that 25–30% of sporadic breast cancers show loss of heterozygosity at these loci (16, 23–26). This raises the intriguing possibility that the normal functions of these genes are temporally and/or developmentally restricted.

Recently, important clues to BRCA1 and BRCA2 function have come from biochemical studies demonstrating that treatment of cells with a variety of DNA-damaging agents leads to the rapid phosphorylation of BRCA1 (27, 28). Moreover, both BRCA1 and BRCA2 have been shown to directly or indirectly bind to RAD51, a homologue of RecA that has been implicated in DNA repair and recombination (29–32). These and other observations have led to the hypothesis that BRCA1 and BRCA2 are involved in the cellular response to DNA damage. Consistent with this hypothesis, embryonic cells from mice homozygous for mutations in the *Brca2* locus have an increased sensitivity to DNA-damaging agents (30, 33, 34). It is interesting to speculate that the developmental regulation of *BRCA1* and *BRCA2* expression or function may contribute to the age-dependent susceptibility of the breast to ionizing radiation-induced carcinogenesis described above.

The markedly elevated risk of breast cancer observed in women carrying germ-line mutations in *BRCA1* and *BRCA2* strongly suggests that these gene are critical for the properly regulated growth of mammary epithelial cells. As a first step toward understanding the developmental role of *BRCA1* and *BRCA2*, we have analyzed the spatial and temporal expression of the murine homologues of these genes during embryogenesis, in the mammary gland during postnatal development, and in adult tissues (35, 36). These studies reveal that expression of both *Brca1* and *Brca2* are tightly regulated during mammary gland development. For example, *Brca1* and *Brca2* expression levels in the mammary glands of adolescent female mice undergoing ductal morphogenesis are significantly higher than those found in the mammary glands of mature females in whom ductal morphogenesis has been completed (35, 36). This temporal pattern of expression is explained in part by the observation that *Brca1* and *Brca2* are expressed at high levels in terminal end buds, which are puberty-specific structures that contain rapidly proliferating cells undergoing differentiation (35–37). *Brca1* and *Brca2* mRNA levels are also markedly up-regulated in the mammary gland early in pregnancy, a period during which alveolar buds begin the process of rapid proliferation and differentiation to form mature, milk-producing alveoli (35–38). This up-regulation of *Brca1* and *Brca2* expression occurs preferentially in developing alveoli as compared to adjacent epithelial ducts, consistent with patterns of proliferation (35, 36). Indeed, at virtually all stages of development, *Brca1* and *Brca2* expression are restricted to cellular compartments actively involved in proliferation and differentiation. These patterns of expression suggest that these tumor suppressor genes may play a role in the normal development of the breast and other tissues.

The spatial and temporal patterns of *Brca1* and *Brca2* expression during development likely reflect the fact that expression of these genes is tightly regulated as a function of proliferation. We have shown that *Brca1* and *Brca2* mRNA levels are high in exponentially growing cells and low in quiescent cells (39). During progression through the cell cycle, *Brca1* and *Brca2* mRNA levels increase during G_1 and attain maximal levels at the G_1-S transition (39). Similar observations have been made for human BRCA1 and BRCA2 at both the mRNA and protein levels (39–46). These findings clearly demonstrate that proliferative stimuli modulate the expression of these genes. Despite the strong correlation between *Brca1* and *Brca2* expression and proliferative status, the expression of these genes also appears to be influenced by factors other than proliferation. For example, we have shown that *Brca1* and *Brca2* mRNA levels are coordinately up-regulated in postconfluent HC11 mammary epithelial cells during differentiation as well as following treatment with insulin and glucocorticoids (39). *Brca1* and *Brca2* expression increase in this setting to levels as high as those found in actively proliferating cells, despite the fact that cellular proliferation rates remain low under these experimental conditions. Together, these observations imply that *Brca1* and *Brca2* may be involved in the processes of proliferation and differentiation in the breast.

A particularly intriguing finding of our studies has been the striking degree to which *Brca1* and *Brca2* are temporally and spatially coexpressed at the mRNA level (36). We have found that *Brca1* and *Brca2* are expressed at similar levels in a similar set of tissues and in similar cellular compartments within those tissues. In fact, the developmental expression patterns of these two putative tumor suppressor genes are essentially identical during embryogenesis and in multiple tissues of the adult. This similarity is particularly evident during postnatal mammary gland development as *Brca1* and *Brca2* expression are each up-regulated during puberty and pregnancy. The coordinate induction of these genes in proliferating and differentiating mammary epithelial cells *in vitro* may provide a cellular basis for this similarity (39). These findings suggest that similar pathways and stimuli regulate the expression of *Brca1* and *Brca2* in multiple cell types. Taken together with the fact that inherited mutations in either *BRCA1* or *BRCA2* predispose mammary epithelial cells to transformation, the striking similarities in *Brca1* and *Brca2* expression patterns formed the initial basis for speculation that these genes may function in overlapping pathways and may even directly interact.

As alluded to above, no somatic mutations have been identified in *BRCA1* or *BRCA2* in sporadic breast cancers. This puzzling observation could be explained if the function of these cancer susceptibility genes in the mammary gland were restricted to specific developmental stages, as might be suggested by the tightly regulated expression that these molecules exhibit during mammary gland development. Similarly, in light of the proposed relationship between normal mammary gland development and reproductive risk factors for breast cancer, it is interesting to note that *Brca1* and *Brca2* are each up-regulated in the breast during puberty and pregnancy because these stages of development are each associated with increases in cellular proliferation as well as increases in breast cancer risk. Potentially, the induction of *Brca1* and *Brca2* expression during these developmental stages may be a protective response to proliferation or to DNA damage that accompanies proliferation, as suggested by the observation that *Rad51* is also up-regulated in proliferating cells (35, 47).

Our laboratory has chosen to focus on understanding BRCA1 and BRCA2 function in mammary epithelial cells because considerably

less is known about their function in this context and because breast cancer is the most important clinical phenotype associated with germ-line mutations in these genes. Specifically, we are interested in those aspects of mammary gland biology responsible for the observation that women carrying germ-line mutations in *BRCA1* and *BRCA2* preferentially develop cancer of the breast. Because this may ultimately relate to mammary-specific functions of these molecules, a complete understanding of the role played by these genes in breast cancer susceptibility will almost certainly require that their functions be studied directly in the mammary epithelium. As such, we are analyzing the impact of altering *BRCA1* and *BRCA2* expression levels on proliferation, differentiation, and DNA repair in the mammary epithelium using *in vivo* and *in vitro* model systems. These studies may provide insight into mechanisms of growth control and DNA damage response in normal mammary epithelial cells as well as serve as a foundation for understanding how the absence or mutation of these molecules promotes carcinogenesis.

Novel Protein Kinases

A second approach to investigating the relationship between development and carcinogenesis in the breast is to study members of a family of regulatory proteins that are typically involved in differentiation, development, and carcinogenesis. Analysis of these processes in a variety of model systems has underscored the key role frequently played by protein kinases. Many protein kinases function as intermediates in mitogenic signal transduction pathways or encode growth factor receptors whose overexpression, aberrant expression, or mutation to ligand-independent activated forms results in transformation. Several members of the protein kinase family have been shown to be involved in the development of breast cancer both in humans and in rodent model systems including the epidermal growth factor receptor, the insulin-like growth factor-I receptor, the fibroblast growth factor receptor family, HER2/Neu, Met, and Src. For instance, amplification and overexpression of *HER2/Neu* and *EGFR* have each been correlated with aggressive tumor phenotype and poor clinical prognosis. Similarly, overexpression of certain protein kinases or of their ligands in transgenic animals results in malignant transformation of the mammary epithelium. To date, however, evidence for a causal role of protein kinases in the initiation and progression of breast cancer exists for only a few members of this family of proteins. For this reason, we embarked on a screen designed to identify tyrosine kinases and serine-threonine kinases expressed in the murine breast during normal development and in breast cancer.

First-strand cDNA was prepared from mRNA isolated either from mammary glands of mice at specific developmental stages or from a series of mammary epithelial cell lines derived from breast tumors that arose in transgenic mice expressing either the activated *neu*, c-*myc*, H-*ras*, or *int2* oncogenes (48–50). Degenerate PCR was used to amplify kinase catalytic subdomains VI–IX, and the resulting cDNA clones were screened to identify those harboring catalytic domain fragments of protein kinases (51–53). This screen identified 41 kinases: 33 tyrosine kinases and 8 serine-threonine kinases, 3 of which are novel.[4] We have characterized the temporal and spatial expression of these kinases during mammary gland development as well as in a panel of mammary epithelial cell lines derived from breast tumors arising in transgenic mice expressing either the activated *neu*, c-*myc*, H-*ras*, or *int2* oncogenes.[4] This analysis has revealed that many of these kinases are preferentially expressed in the breast during specific stages of puberty, pregnancy, lactation, and postlactational regression.

Our laboratory has subsequently focused on the function of three novel serine-threonine kinases identified in our screen: *Hunk*, *Punc*, and *Krct*. The novel protein kinase, *Hunk*, was initially isolated from a mammary epithelial cell line derived from a breast tumor that arose in a transgenic mouse expressing the *neu* oncogene (54).[4,5] Analysis of sequence homology within a portion of the catalytic domain of *Hunk* suggests that it is a serine/threonine kinase with highest homology to the *SNF1* kinase family. The novel protein kinase, *Punc*, was initially isolated from the mammary glands of mice undergoing early postlactational regression.[4,6] The catalytic domain of *Punc* is 60% identical at the amino acid level to calcium/calmodulin-dependent protein kinase I and shares a lower homology with other members of the calcium/calmodulin-dependent kinase family (55).[6] *Krct* appears to represent a new family of mammalian protein kinases and is most closely related to a protein kinase recently identified by the yeast genome project that does not fall into any of the families of protein kinases previously identified in yeast (54).

Hunk and *Punc* appear to be particularly relevant to studies of the relationship between mammary gland development and carcinogenesis by virtue of their patterns of expression.[7,8] Specifically, *Hunk* is expressed at low levels in the mammary glands of immature and mature virgin animals and undergoes a dramatic up-regulation of expression during early pregnancy. *Hunk* expression rapidly drops to basal levels by midpregnancy and decreases further during lactation and early postlactational regression. Like *Hunk*, *Punc* expression is also up-regulated in the mammary epithelium during pregnancy. However, unlike *Hunk*, maximum levels of *Punc* expression occur late in pregnancy just prior to parturition.

To determine whether the developmental changes in *Hunk* and *Punc* expression observed during pregnancy represent global changes in expression occurring throughout the mammary gland or changes in the abundance of an expressing subpopulation of cells, we have defined the spatial pattern of expression of these kinases.[7,8] This was of particular interest because the expression of several protein kinases has been shown to be cell lineage restricted, thereby permitting their use as markers for biologically interesting subpopulations of cells. Examination of the spatial pattern of *Hunk* and *Punc* expression revealed that throughout the course of mammary development both kinases are expressed predominantly in the mammary epithelium. Interestingly, the expression of each of these kinases in the mammary epithelium is strikingly heterogeneous, with the greatest number of *Hunk*-expressing cells being observed at day 7 of pregnancy and the greatest number of *Punc*-expressing cells being observed at day 20 of pregnancy. This pattern of expression does not appear to be due to the heterogeneous distribution of cells through the cell cycle. Analogously, studies of the expression of these kinases in a variety of other tissues suggest that *Hunk* and *Punc* expression may also identify subsets of cells in other organs besides the breast. These observations suggest that *Hunk* and *Punc* are differentially expressed in distinct

[4] L. A. Chodosh, H. P. Gardner, J. V. Rajan, D. B. Stairs, S. T. Marquis, and P. Leder, Protein kinase expression during mammary gland development, manuscript in preparation.

[5] H. P. Gardner, J. V. Rajan, S. T. Marquis, and L. A. Chodosh, Cloning and characterization of a novel *SNF1*-related serine/threonine kinase, *Hunk*, manuscript in preparation.

[6] H. P. Gardner, J. V. Rajan, S. T. Marquis, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, and L. A. Chodosh, Cloning and chromosomal localization of a novel CaM kinase, *Punc*, manuscript in preparation.

[7] H. P. Gardner, S. I. Ha, S. T. Marquis, and L. A. Chodosh, Spatial and temporal regulation of *Hunk* expression during normal mammary gland development, manuscript in preparation.

[8] H. P. Gardner, S. I. Ha, and L. A. Chodosh, Differentiation-dependent expression of a novel calcium-calmodulin-dependent protein kinase, *Punc*, in the murine breast, manuscript in preparation.

epithelial cell subtypes in the breast that are differentially regulated during pregnancy.

To further investigate this hypothesis, we have examined *Hunk* and *Punc* expression in a panel of mammary epithelial cell lines derived from independent mammary adenocarcinomas arising in transgenic mice expressing the *neu,* c-*myc*, H-*ras*, or *int2* oncogenes.[7,8] Surprisingly, all eight cell lines derived from breast tumors that arose in transgenic mice expressing the *neu* or H-*ras* oncogenes were found to express high levels of *Hunk* mRNA, whereas none of the seven cell lines derived from breast tumors that arose in transgenic mice expressing the *c-myc* or *int-2* oncogenes expressed detectable levels of *Hunk*. Conversely, all seven cell lines derived from breast tumors that arose in transgenic mice expressing the *c-myc* or *int-2* oncogenes expressed *Punc* mRNA, whereas none of the eight cell lines derived from breast tumors that arose in transgenic mice expressing the *neu* or H-*ras* oncogenes expressed detectable levels of *Punc*. In each case, kinase expression levels observed in tumor cells were significantly higher than those observed in nontransformed mammary epithelial cells.

The heterogeneous spatial patterns of *Hunk* and *Punc* expression in the breast, along with the mutually exclusive patterns of expression of these two kinases in transgenic mammary epithelial cell lines, suggest that these novel serine-threonine kinases may be differentially expressed in distinct mammary epithelial cell subtypes that are themselves differentially regulated during pregnancy. The observation that *Hunk* and *Punc* are overexpressed in cell lines derived from breast cancers induced by the *neu* or c-*myc* oncogenes, respectively, suggests either that *Hunk* and *Punc* are downstream targets of the *neu* and c-*myc* oncogenes or that these kinases identify epithelial cell subtypes that are preferentially transformed either by *neu* or c-*myc*.

Each of these hypotheses is based on our observations suggesting that the normal mammary epithelium appears to be composed of distinct *Hunk*- and *Punc*-expressing cell types. The first hypothesis postulates that *Hunk* mRNA expression is activated by the *neu* and/or H-*ras* pathways, whereas *Punc* mRNA expression is activated by the c-*myc* and/or *int2* pathways. In this model, *neu* (or c-*myc*) transgene expression in the mammary epithelium induces *Hunk* (or *Punc*) expression in all mammary epithelial cell types that express the transgene. As a consequence, tumors that arise from the epithelium display the same differential pattern of expression exhibited by the parental normal transgenic mammary epithelium. The second hypothesis postulates that *neu* and c-*myc* preferentially transform two different mammary epithelial cell types, one of which (in the case of *neu*) is marked by *Hunk* expression and the other of which (in the case of *c-myc*) is marked by *Punc* expression. In this model, overexpression of *Hunk* in *neu*-induced tumors reflects the selection and outgrowth of an *Hunk*-expressing epithelial cell subtype that otherwise represents a minor fraction of cells in the normal mammary epithelium. That is, *Hunk* and *Punc* expression may be restricted to distinct epithelial cell subtypes that are preferentially transformed by these oncogenes.

Our data suggest that the novel serine/threonine kinases identified in our laboratory may serve as markers for biologically interesting subpopulations of epithelial cells in the breast that are relevant both to development and carcinogenesis. Current work in our laboratory on *Hunk*, *Punc*, and *Krct* focuses on placing these kinases in known or novel signal transduction pathways and on determining their role in mammary development and carcinogenesis using transgenic and knockout animal models as well as tissue culture model systems. In addition, we have cloned the human homologues for each of these genes and are currently determining whether *Hunk*, *Punc*, and *Krct* are mutated, amplified, or overexpressed in human tumors or tumor cell lines.

Parity-induced Changes in the Breast

A third approach that our laboratory is taking to explore the relationship between development and carcinogenesis in the breast is to focus on the molecular and cellular changes that occur in the breast as a result of reproductive events known to influence breast cancer risk. Epidemiological studies have consistently shown that women who undergo an early first full-term pregnancy have a significantly reduced lifetime risk of breast cancer (1, 56–64). This association is independent of parity (*i.e.*, number of live births). In contrast, women who undergo their first full-term pregnancy after the age of 30–35 years appear to have a risk of breast cancer that is actually higher than that of nulliparous women. This suggests that parity-induced protection against breast cancer is principally dependent upon the timing of a first full-term pregnancy rather than on its occurrence *per se*. These observations imply that an early first full-term pregnancy results in a change in the breast, either directly or indirectly, that confers a decreased risk for the subsequent development of breast cancer. Because aborted pregnancies are not associated with a decreased risk for breast cancer, it has been hypothesized that the protective effect of parity requires attaining the terminally differentiated state of lactation (2, 3, 6, 59, 65–71). Unfortunately, the biological basis of parity-induced protection against breast cancer is unknown. In principle, the protective effect of early first childbirth could result from the pregnancy-driven terminal differentiation of a subpopulation of target cells at increased risk for carcinogenesis, from the preferential loss of a subpopulation of target cells during postlactational regression or from a permanent systemic endocrine change affecting the breast in such a way as to reduce the risk of carcinogenesis. Clearly, a more thorough elucidation of the molecular and cellular changes that take place in the breast as a result of parity will be required to fully understand this phenomenon.

The realization that specific reproductive endocrine events alter breast cancer risk in a predictable fashion raises the possibility that events known to decrease breast cancer risk might be mimicked pharmacologically. The desire to pursue this objective is heightened by the fact that, although it is now possible by genetic means to identify women who are at elevated risk for developing breast cancer, interventions between the extremes of more frequent mammographic screening and prophylactic bilateral mastectomy are only now beginning to be considered. As such, reducing breast cancer risk via hormonal manipulations designed to mimic naturally occurring endocrine events could represent a feasible alternative. It is to this end that both early first full-term pregnancy and early menopause have been proposed as logical paradigms on which to model the hormonal chemoprevention of breast cancer. The achievement of this goal, however, has been hampered by current ignorance regarding the mechanism by which reproductive history alters breast cancer risk. As such, the rational design of hormonal chemoprevention regimens would benefit from a better understanding of the influence of development on breast cancer risk. An additional stumbling block in the development of chemoprevention regimens aimed at reducing breast cancer risk has been the prolonged and costly clinical trials required to determine the efficacy of these regimens due to reliance on the development of breast cancer as a clinical end point (72–75). As such, the identification and use of intermediate molecular end points that accurately identify changes in the breast associated with changes in breast cancer risk would facilitate the development of such chemopreventive regimens. To this end, we have chosen to exploit the relationship between development and carcinogenesis in the breast to generate rational and biologically plausible candidate surrogate end point biomarkers.

The mechanism of parity-induced protection against breast cancer

is likely to involve complex genetic and epigenetic processes that may be influenced by reproductive endocrine variables as well as by inherited genotypes. In this context, it is useful to analyze complex processes such as this in model systems that recapitulate relevant epidemiological findings, permit critical aspects of reproductive history to be rigorously controlled, reduce genetic variation, and permit the examination of molecular and cellular events at defined developmental stages of interest in normal tissue. The use of animal models to study the impact of mammary gland development on breast cancer risk is facilitated by the fact that the structure, function, and developmental stages through which the mammary gland passes are similar in humans and in rodents (76, 77). Administration of the carcinogen DMBA to nulliparous Sprague Dawley rats induces mammary adenocarcinomas that are hormone dependent and histologically similar to human breast tumors. In contrast, rats that have previously undergone a full-term pregnancy are highly resistant to the induction of breast cancer by carcinogen administration, as compared with age-matched nulliparous controls (2, 6, 78–83).

Paralleling these functional differences, there are also marked morphological differences between the adult nulliparous mammary gland and the mammary glands of age-matched parous littermates that have undergone a single cycle of pregnancy, lactation, and regression. These parity-induced morphological changes are permanent because nulliparous and parous glands may be distinguished easily even after 1 year of postlactational regression (3).[9] Similar morphological changes are also seen in mice and in rats and are analogous to those reported in the parous human breast (70, 77). These observations support the hypothesis that parity results not only in a permanent change in the functional state of the breast (*i.e.*, susceptibility to carcinogenesis) but also in permanent structural changes in the breast. Finally, the fact that the Sprague Dawley DMBA model system mirrors complex epidemiological phenomena observed in humans, and that numerous molecules believed to play important roles in the pathogenesis of human breast cancer have similar effects in rodents, suggests that rodent model systems such as this can be a valuable tool for understanding fundamental aspects of mammary gland biology and breast cancer etiology.

We hypothesize that understanding the impact of parity on breast cancer risk will require a thorough understanding of the manner in which reproductive history affects subpopulations of cell types present in the breast. To address this hypothesis, we are using rodent model systems to identify and evaluate genes that are differentially expressed in the breast as a function of parity. Candidate genes that are specifically expressed in either the parous or the nulliparous rodent breast are being isolated and identified using a variety of approaches. These differentially expressed genes are being used as biomarkers for the cellular and molecular changes that occur in the breast as a result of an early first full-term pregnancy to define the impact of early parity on the development and differentiation of specific cell types in the breast. Finally, biomarkers that are found to be biologically informative in the rodent model system are being tested for their ability to detect parity-associated changes in histologically normal breast tissue obtained from nulliparous and parous women with known reproductive history and hormone exposures. The level and spatial pattern of expression of each of these candidate biomarkers is being analyzed in human tissue and evaluated with respect to parity as well as other parameters of reproductive endocrine history, such as age, age at first full-term pregnancy, menopausal status, and exogenous hormone use. These studies will determine whether candidate biomarkers characterized in rodent model systems can specifically detect parity-induced changes in the human breast.

To date, this approach has yielded a variety of genes that are expressed at higher levels in the mammary glands of parous animals as compared with age-matched virgin controls, confirming the utility of this approach for isolating genes that are specifically expressed in the breast as a function of reproductive history.[9] Several of the parity-specific genes that we have initially isolated are markers of mammary epithelial cell differentiation, such as milk proteins. This finding suggests that the parous breast is more "differentiated" than the nulliparous breast and, as such, is consistent with the proposal made by Russo and Russo (2, 84) that parity protects against breast cancer by virtue of the differentiation that it induces. The developmental patterns of expression of milk protein genes are notably heterogeneous because each is up-regulated at a specific point in the alveolar differentiation pathway (85). Interestingly, we have found that the expression patterns of several of these genes reflect subtle aspects of reproductive history.[9] As such, studying the regulated expression of this class of genes as a function of reproductive history may provide insights into parity-related events in the breast. In addition, we have isolated a number of genes that are as yet unidentified. Given their interesting developmental patterns of regulation and parity-specific pattern of expression, these genes appear to represent an informative pool of candidate biomarkers for detecting changes in the breast associated with reproductive events.

In theory, the parity-specific pattern of expression for a given biomarker could reflect a global increase in expression of the gene in all mammary epithelial cells, an increase in the percentage of expressing cells in the breast, or both. We are analyzing the developmental pattern of expression of candidate genes by *in situ* hybridization to distinguish between these mechanisms. Our results indicate that parity-specific patterns of expression for different genes result from distinct developmental pathways. For example, these studies reveal examples of parity-dependent global changes in expression as well as parity-dependent changes in the abundance of expressing cells. This latter example is suggestive of a permanent pregnancy-induced expansion in the number of cells expressing a given biomarker in the breast.[9] These findings are consistent with the hypothesis that reproductive events may permanently alter the biology of the breast by differentially affecting subpopulations of cells.

We have also determined the impact of several reproductive parameters on the differential pattern of expression of these genes.[9] These experiments reveal that the parity-specific pattern of expression for some genes is independent of age, duration of postlactational regression, and age at first full-term pregnancy. In contrast, other genes we have identified are expressed in a parity-specific manner in the mammary glands of animals that have been mated as adolescents but not in the mammary glands of animals that have been mated as adults. These results suggest that the regulation of expression of such genes reflects developmental events in the mammary gland that are specific for age at first full-term pregnancy. These findings suggest that candidate cDNA biomarkers generated by these approaches may provide insight into subtle aspects of the molecular and cellular changes that occur in the breast as a result of parity. Ultimately, these studies are intended to gain sufficient understanding of the molecular pathways responsible for parity-induced protection against breast cancer in order to permit this naturally occurring protective event to be mimicked pharmacologically.

Summary

The current aims of this laboratory are designed to develop the molecular tools required to understand the relationship between nor-

[9] C. M. D'Cruz, J. Wang, S. I. Ha, and L. A. Chodosh, Reproductive history results in a permanent change in the expression of specific genes in the murine breast, manuscript in preparation.

mal mammary gland development and mammary carcinogenesis, as reflected in the epidemiology of reproductive endocrine risk factors for breast cancer. We have taken three approaches toward understanding this relationship, including: determining the role normally played by breast cancer susceptibility genes in mammary epithelial development; studying the function of three novel protein kinases in the breast; and identifying and analyzing genes that are specifically expressed in the breast during developmental stages associated with changes in breast cancer risk. We anticipate that these approaches will ultimately lead to a clearer understanding of the mechanisms by which breast cancer susceptibility is modulated by reproductive history.

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Discussion

Dr. Andrew Feinberg: I have a really simple-minded question. These are very elegant studies, but I worry a bit about transgene-induced tumors, because in a sense you're starting with loaded dice. Aren't there any models of spontaneous mammary tumorigenesis? I thought there were some dogs or other species that developed cancer in a similar epidemiological manner as you mentioned for humans at the beginning. But, I don't know this field, so I may be totally wrong.

Dr. Chodosh: It is true that there are certain breeds of dogs that do develop breast cancer spontaneously, though I am not aware of any that show parity-induced protection against breast cancer. Obviously, how you choose a model to study a particular question is a central issue. Regarding transgenic rodent models of breast cancer there are a couple of points worth making.

The first is that our main experimental thrust is to look at the normal developmental biology of the breast. There is no question that the developmental stages through which the breast passes for both the mouse and the rat are exceedingly similar to what one finds in the human. That is, the developmental processes are as highly conserved as histology and tissue architecture.

The second is that it's quite clear from transgenesis experiments that many of the pathways that are altered during the process of carcinogenesis in the human breast cause similar problems in the rodent breast when altered by transgenic approaches. That is, the molecular pathways involved are highly conserved. So, while tumor development in a transgenic system is not "spontaneous" in the same way that we think of for human breast cancers, I would argue that the

history of cancer biology suggests that they are still quite useful models to examine pathways involved in development and carcinogenesis. So at the moment, as far as animals that we can work with, particularly those that we can genetically manipulate, we have mice. Similarly, in the rat, one is somewhat restricted to carcinogen-induced models, which may or may not faithfully mimic the processes involved in human carcinogenesis.

We think about the suitability of our model systems a great deal, and it's not clear to me that there's another *in vivo* system available at the present time that's more appropriate.

Speaker: Do you have any evidence these kinases play similar roles in the human breast? Because human breast cancer is quite different. Pathological studies are quite different from real breast cancer, because it's quite complicated by different pathways. So, my interest at the moment is that even if we are able to link these kinases to the set of human reactants, it is different with different types of breast cancer and different kinases being expressed. How do you plan to address these potential differences?

Dr. Chodosh: A very important question, which explains why we are moving into human tissue and human breast cancer cell lines to address some of these issues. This is information that we're currently gathering. The data that I showed you in human breast cancers and cancer cell lines are quite recent, so it's too preliminary for us to know whether there is some correlation between the expression of our kinases and Erb2 status or ER status, or a particular histological cell type. Regarding tumors that are marked by *Hunk* or *Punc* expression, clearly we would want to know whether they behave differently in terms of patient prognosis or response to therapy. We don't know that yet, though that's certainly something that we're very interested in.

Dr. Robert Ryan: I would like to ask, have you considered perhaps doing something like the chip-based assay where now you use the MMTV-neu and MMTV-c-myc breast cancer cell lines and test those samples for changes by looking at the various genes that are up-regulated or down-regulated. It might give you a handle on that, do you think?

Dr. Chodosh: Yes, that's certainly a possibility. In the context of DNA chip technology, I think we'd probably want to make the fewest possible changes that we could, starting with the most normal cells we can, then induce expression of a *Hunk* or *Punc* transgene and ask what genes are downstream, as opposed to using as a starting point tumor cell lines that obviously have undergone many unrelated changes over the long period of time they have been in culture. Certainly, I agree it's an important new technology.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Encl